CADMIUM: A TOXIN AND A NUTRIENT FOR MARINE PHYTOPLANKTON

by

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JENNIFER GRANT LEE

Submitted to the MIT/WHOI Joint Program in Chemical Oceanography
on May 5, 1995 in partial fulfillment of the requirements for the degree
of Doctor of Philosophy in Chemical Oceanography

Abstract

Although cadmium is known to be very toxic, it exhibits nutrient-like vertical concentration profiles in the open ocean. Here I show that cadmium is a nutrient for the marine diatom *Thalassiosira weissflogii*, a chlorophyte and some prymnesiophytes at inorganic zinc and cadmium concentrations typical of surface sea water (although cadmium cannot completely replace zinc). Very low concentrations of inorganic cadmium (5pM) that are beneficial under conditions of moderate zinc limitation (3pM) become toxic in cultures severely limited by zinc (0.2pM). The role of cadmium as an algal nutrient is thus observable in a narrow, species-specific range of inorganic zinc and cadmium concentrations.

Detailed studies of *T. weissflogii* show that over a wide range of external inorganic cadmium (5-500pM) and inorganic zinc (2-16pM) concentrations, cadmium uptake kinetics in *T. weissflogii* are regulated, the maximum uptake rate increasing with decreasing external metal concentrations. The intracellular cadmium quota is maintained at relatively constant levels over this range. At high inorganic cadmium concentrations (5nM), export of cadmium, most likely complexed to the metal-binding polypeptide phytochelatin, also regulates intracellular cadmium concentrations. The efflux of both cadmium and phytochelatin stops when the external inorganic cadmium concentration is reduced to "natural" levels ($\leq$7pM). The intracellular pool of cadmium-phytochelatin complex serves as a source of Cd to proteins after the external Cd supply is cut off. The cadmium-phytochelatin complex is not very stable once outside the cell since the exported cadmium appears to be available to *T. weissflogii*.

The same low level of inorganic cadmium that enhances the growth rate of zinc-limited cells restores the activity of carbonic anhydrase, thought to be the key enzyme limiting growth of *T. weissflogii* at low zinc. Cadmium coelutes with at least one of the multiple isoforms of carbonic anhydrase produced by *T. weissflogii* and covaries with activity of this isoform (which in turn depends on PCO_2). Cadmium may therefore play an essential role in carbon uptake under conditions of zinc limitation. The substitution of cadmium for zinc in carbonic anhydrase links the geochemical cycle of cadmium to those of zinc and carbon.

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Finally, I am deeply grateful to my family, Richard, Jacqueline and Debby Lee, who have always tolerated my foibles and nurtured my enthusiasms. I would also like to thank my husband Glenn Moglen, who makes my life joyful and rich and is the best companion in life I could ever wish for.

I would like to dedicate my thesis in memory of my father, Richard Dooban Lee.

Biographical note

Education

MIT/WHOI Joint Program in Oceanography, Cambridge, MA. (1989-1995)
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Research Analyst, Gradient Corp., Cambridge, MA. 1987-1989.
Critiqued sampling plans, evaluated analytical work, and performed exposure estimates for hazardous waste sites.

Research Analyst, JACA Corp., Fort Washington, PA. 1986-1987.
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Publications

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Lee, J. G. and F. M. M. Morel. In press. "Replacement of zinc by cadmium in marine phytoplankton." Mar. Ecol. Prog. Ser.

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Abbreviations and Definitions

CA	carbonic anhydrase
Cd′	inorganic Cd concentration
EC	γ-Glu-Cys subunits of phytochelatin = 2 x (phytochelatin, n=2) + 3 x (phytochelatin, n=3) + 4 x (phytochelatin, n=4)
isoforms	proteins which have the same biochemical function but differing amino acid sequences
Cd quota	Cd concentration per cell.
Zn′	inorganic Zn concentration

Chapter 1 Introduction

Background

The concentration of dissolved Cd in open ocean waters has a nutrient-type profile, increasing with depth from very low values at the surface to reach high, fairly constant values below the thermocline (Boyle et al. 1976; Bruland et al. 1978; Bruland and Franks 1983; Martin et al. 1976). Below the thermocline, dissolved Cd concentrations are closely correlated with dissolved phosphate. This correlation of Cd with phosphate has led to the use of the concentration of Cd as recorded in the calciferous tests of foraminifera to estimate past dissolved phosphate concentrations (Boyle 1988).

Price and Morel (1990) proposed that the depletion of Cd from surface seawater might be due to the utilization of Cd by phytoplankton growing in an environment at exceedingly low inorganic Zn (Zn') concentrations. In the laboratory, they found that the coastal diatom *T. weissflogii* was able to grow at near-optimal rates when Cd instead of Zn was added to the culture medium (although at high levels compared to the open ocean). Because of the ubiquitous problem of Zn contamination, these results do not establish that Cd can totally substitute for Zn. Based on the similar size distribution of Cd and Zn in cytoplasmic proteins the authors suggested that the mechanism for enhanced growth by Cd/Zn replacement might be substitution of Cd for Zn in one or more metalloenzymes.

The key enzyme in Zn limitation of *T. weissflogii* appears to be carbonic anhydrase (Morel et al. 1994). Reduced carbonic anhydrase (CA) activity apparently limits growth rate because of the role of the enzyme in inorganic carbon uptake. Cadmium (as well as cobalt) supplementation restores at least some of the CA activity in Zn-limited cultures, but we do not know if this is due to direct substitution of Cd for Zn in CA, to the synthesis of a distinct Cd-CA enzyme, or to displacement of Zn from other metalloproteins.

Inorganic Co has also been shown to partially restore growth of Zn-limited cultures of *T. weissflogii* (Price and Morel 1990). Cobalt appears to restore growth by replacing Zn in carbonic anhydrase (Yee and Morel in review). To simplify the study of Cd/Zn replacement, inorganic Co was omitted from the culture medium in all experiments in this thesis. The culture medium did contain 0.4 nM of vitamin B_{12}, but this source of Co has been shown to be insufficient to satisfy demands for either inorganic Zn or Co in several species of marine phytoplankton (Yee and Morel in review, Sunda and Huntsman in press). This thesis might therefore be more properly described as studying the role of Cd in alleviating Co-Zn co-limitation.

Cadmium is also one of the most toxic trace metals and is found in high concentrations in wastes from Zn smelting, electroplating and sewage treatment. In animals, the main cellular response to Cd stress is the production of metallothionein, a primary gene product with a high cysteine content, whose role is to complex the free metal. Instead of metallothionein, algae and higher plants synthesize low molecular-weight, cysteine-rich polypeptides known as phytochelatins. Phytochelatins are a family of oligomers with the structure $(\gamma\text{-Glu-Cys})_n\text{Gly}$, where n varies from 2 to 11 (Grill et al. 1987, Gekeler et al. 1988). They are synthesized from glutathione (which has the structure of phytochelatin with n=1), by the enzyme γ-glutamylcysteine dipeptidyl transpeptidase ("phytochelatin synthase") in response to high concentrations of a wide variety of trace metals and some metalloid oxyanions. Because phytochelatin synthase is activated by high free cellular metal concentrations, phytochelatin production is self-regulating (Grill et al. 1989).

Cadmium is the most effective trace metal for inducing production of phytochelatin in marine phytoplankton. Phytochelatin appears to play a role in metal storage as well as detoxification since low levels are produced by many species of phytoplankton even at inorganic Cd (Cd′) concentrations far below those that impede growth (Ahner et al. in press). At Cd′ concentrations above 0.5 nM, phytochelatin reaches millimolar concentrations in *T. weissflogii* and increases rapidly with Cd′.

Cadmium quotas*, on the other hand, are much more constant resulting in cellular phytochelatin to Cd ratios of over 100 at nanomolar Cd′ concentrations. Phytochelatin is induced very rapidly in *T. weissflogii* by exposure to Cd. Removing conditions of Cd stress results in rapid restoration of low, constitutive phytochelatin levels in the cell (Ahner and Morel in press).

*The cellular Cd quota is the Cd concentration per cell.

Overview of thesis

Cd enhances the growth of Zn-limited cultures of *T. weissflogii* at Cd′ and Zn′ levels which are close to those that have been measured in the surface water of the open ocean (Bruland 1989, 1992) although Cd cannot completely replace Zn (Chapter 2, Lee et al. in press). Low Cd′ and Zn′ concentrations were achieved by growing cultures in media buffered with EDTA. Only the inorganic fraction (i.e. non-EDTA fraction) of the these trace metals is available to phytoplankton because of the slow time scale for dissociation of their EDTA complexes relative to diffusion to the cell (Price et al. 1988/9). Buffering inorganic trace metal concentrations with EDTA has the further advantage that any chelators released over time by the phytoplankton themselves have a negligible effect on trace metal speciation in the medium.

Over a wide range of external Cd′ (5-500 pM) and Zn′ (2-16 pM) concentrations*, Cd uptake kinetics are regulated, the maximum uptake rate increasing with decreasing external metal concentrations. The intracellular Cd quota is maintained at relatively constant levels over this range (details of the method used to measure Cd quotas are given in Chapter 6). The same low level of Cd′ (5 pM) that enhances the growth rate of Zn-limited cells restores the activity of carbonic anhydrase, which is thought to be the key enzyme limiting growth of *T. weissflogii* at low Zn′ (Morel et al. 1994). Cadmium also coelutes with some of the isoforms† of carbonic anhydrase detected by a post-electrophoresis enzyme assay indicating that Cd substitution in carbonic anhydrase is likely partly responsible for the nutritional role of Cd.

We extend our study of Cd/Zn replacement to other species of marine phytoplankton (Chapter 3, Lee and Morel in press). Cadmium can also enhance the growth of a wide range of species when they are Zn-limited including a chlorophyte and some prymnesiophytes. Cadmium was a nutrient under Zn limitation for half the

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* These Cd′ and Zn′ concentrations ranges were selected both to include measured Cd′ and Zn′ concentrations in the oligotrophic ocean (Bruland 1989, 1992) and to reflect the physiological responses of *T. weissflogii* to given concentrations of Cd′ and Zn′ (e.g. the lowest Cd′ which increased growth and the Zn′ levels which limited growth).

† Isoforms are proteins which have the same biochemical function but differing amino acid sequences.

species studied. The replacement of Cd by Zn occurs at environmentally relevant Cd′ and Zn′ concentrations (5 pM Cd′ and 0.2-3 pM Zn′). As in the study of *T. weissflogii* (Chapter 2), low Zn′ and Cd′ concentrations were achieved using culture media buffered with EDTA. Very low concentrations of Cd′ (5 pM) that are beneficial under conditions of moderate Zn limitation (3 pM Zn′) can become toxic in cultures severely limited by Zn (0.2 pM Zn′), however. The role of Cd as an algal nutrient is thus observable in a narrow, species-specific range of Zn′ and Cd′ concentrations.

Some of the physiological effects of Cd toxicity on *T. weissflogii* are quantified in Chapter 4 (Lee et al. in review). At high Cd′ (5 nM), export of Cd plays an important role in regulating the intracellular Cd concentration. The metal-binding polypeptide phytochelatin, which is induced in marine phytoplankton by metal stress (Ahner and Morel in press, Ahner et al. in press, Gekeler et al. 1988), also appears to reduce Cd toxicity by facilitating export of Cd from the cell. Cadmium is likely to be exported as a phytochelatin complex, the stoichiometry of which is ~4 moles of γ-Glu-Cys subunits per mole Cd. Cadmium and phytochelatin export are under physiological control, stopping when the external Cd′ concentration is reduced to "natural" levels (≤7 pM). The Cd-phytochelatin complex is not very stable once outside the cell since the Cd exported is available to *T. weissflogii*.

Finally, in Chapter 5 we test the hypothesis that Cd has a role in carbon acquisition by examining the effect of varying PCO_2 on CA activity and Cd distribution among the cellular proteins of *T. weissflogii*. Growth rates, the concentration of Cd per cell and the fractionation of Cd between the membrane and cytoplasm in the cell are not very different at high, atmospheric, and low CO_2 levels. The amount of Cd coeluting with one of the isoforms of carbonic anhydrase does vary with PCO_2 however, showing that Cd plays a role in carbon uptake under conditions of carbon and Zn co-limitation. There is also a dramatic difference in the cellular phytochelatin

concentration with PCO_2. At low PCO_2 cells contains 1.1 fmol EC*/cell, almost three times as much as is present at high PCO_2, suggesting that phytochelatin serves in the utilization of Cd under CO_2-limited conditions.

* EC = γ-Glu-Cys subunits of phytochelatin = 2 x (phytochelatin, n=2) + 3 x (phytochelatin, n=3) + 4 x (phytochelatin, n=4)

Conclusions

Cadmium is a nutrient for the marine diatom *T. weissflogii* and a wide range of other species of phytoplankton at Zn′ and Cd′ concentrations typical of surface seawater (although Cd cannot completely replace Zn). In *T. weissflogii*, Cd replaces Zn in at least one isoform of the enzyme carbonic anhydrase, thought to be the key enzyme limiting growth of *T. weissflogii* at low Zn′ (Morel et al. 1994). Cadmium therefore plays a role in carbon acquisition under conditions of Zn limitation.

Cd uptake rates, export of Cd, intracellular Cd quotas and the distribution of Cd in the cell are regulated by *T. weissflogii*. The maximum Cd uptake rate increases with decreasing external metal concentrations so that the intracellular Cd concentration remains relatively constant. Over a wide range of external Cd′ concentrations, the distribution of Cd between proteins in the cell membrane and cytoplasm is also invariant. At very high Cd′ (5 nM), excess Cd is stored as a phytochelatin complex. Export of Cd complexed by phytochelatin from this intracellular pool of excess Cd also helps regulate the Cd quota. In addition, the intracellular pool of Cd-phytochelatin complex can serve as a source of Cd for the cell during growth when external Cd′ is reduced.

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Chapter 2 Cadmium: a nutrient for the marine diatom *Thalassiosira weissflogii*[*]

J. G. Lee, S. B. Roberts and F. M. M. Morel

Abstract

Although cadmium is known to be very toxic, it exhibits nutrient-like vertical concentration profiles in the open ocean. Recent work has shown that under conditions of zinc limitation, cadmium enhances the growth of the marine diatom *Thalassiosira weissflogii.* Here we conclusively demonstrate that cadmium is a nutrient for *T. weissflogii* at inorganic zinc and cadmium concentrations typical of surface seawater. Growth experiments in which cadmium and zinc contamination were scrupulously avoided reveal, however, that cadmium cannot completely replace zinc. Over a wide range of external cadmium and zinc concentrations, cadmium uptake kinetics are regulated and the intracellular cadmium quotas are maintained at relatively constant levels. The same low level of inorganic cadmium that enhances the growth rate of zinc-limited cells restores the activity of carbonic anhydrase, which is thought to be the key enzyme limiting growth of *T. weissflogii* at low zinc. Cadmium also coelutes with some of the isoforms of carbonic anhydrase detected by a post-electrophoresis enzyme assay, indicating that cadmium substitution in carbonic anhydrase is likely partly responsible for the nutritional role of cadmium. The substitution of cadmium for zinc in carbonic anhydrase links the geochemical cycle of cadmium to those of zinc and carbon.

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[*] This chapter is an expanded version of Lee, J. G., S. B. Roberts and F. M. M. Morel. In press. Cadmium: a nutrient for the marine diatom *Thalassiosira weissflogii. Limnol. Oceanogr.*

Introduction

The concentration of dissolved Cd in open ocean waters has a nutrient-type profile with depth (Boyle et al. 1976; Bruland and Franks 1983). Below the thermocline, dissolved Cd concentrations are closely correlated with dissolved phosphate. This correlation of Cd with phosphate has led to the use of the concentration of Cd as recorded in the calciferous tests of foraminifera to estimate past dissolved phosphate concentrations (Boyle 1988).

Price and Morel (1990) proposed that the depletion of Cd from surface seawater might be due to the utilization of Cd by phytoplankton growing in an environment at exceedingly low inorganic Zn concentrations. In the laboratory, they found that the coastal diatom *T. weissflogii* was able to grow at near-optimal rates when instead of Zn Cd was added to the culture medium (although at high levels compared to the open ocean). Because of the ubiquitous problem of Zn contamination, these results do not establish that Cd can totally substitute for Zn. Based on the similar size distribution of Cd and Zn in cytoplasmic proteins the authors suggested that the mechanism for enhanced growth by Cd/Zn replacement might be substitution of Cd for Zn in one or more metalloenzymes.

The key enzyme in Zn limitation of *T. weissflogii* appears to be carbonic anhydrase (Morel et al. 1994). Reduced carbonic anhydrase (CA) activity apparently limits growth rate because of the role of the enzyme in carbon uptake. Cadmium (as well as cobalt) supplementation restores at least some of the CA activity in Zn-limited cultures, but we do not know if this is due to direct substitution of Cd for Zn in CA, to the synthesis of a distinct Cd-CA enzyme, or to displacement of Zn from other metalloproteins.

To definitively establish that Cd is a nutrient for Zn-limited cultures of *T. weissflogii* we study here the kinetics of Cd uptake, the conditions for optimum Cd/Zn substitution, including whether substitution might occur at open ocean levels of inorganic Cd and Zn, and the biological role of Cd.

Materials and Methods

Culture Medium

Cultures of *T. weissflogii* clone ACTIN (Center for the Culture of Marine Phytoplankton, Bigelow Laboratory) were grown in synthetic ocean water prepared according to the recipe for Aquil (Price et al. 1988/89) with the following modifications: inorganic cobalt was omitted from the recipe since cobalt, like cadmium, can substitute for zinc (Price and Morel 1990); Zn varied; and Cd was added. Inorganic trace metal concentrations, M′, were calculated from total concentrations using the computer program MINEQL (Westall et al. 1976). The ratio of hydrated Zn ions, $[Zn^{2+}]$, to inorganic zinc was calculated to be 0.82. The ratio of $[Cd^{2+}]$ to inorganic cadmium was much lower, 0.029, due to cadmium complexation by chloride ions. Radiolabelled Cd was obtained from New England Nuclear or Amersham (carrier-free) and added as a unsterilized stock in 0.01 N HCl. In experiments using radiolabelled Cd, the total EDTA was lowered from 100 μM to 10 μM to reduce the radioactivity necessary*. Total trace metal concentrations were adjusted accordingly and inorganic trace metal concentrations recalculated using MINEQL.

Media were allowed to equilibrate for at least 12 hours after addition of the EDTA trace metal mix and at least 6 hours after Zn or Cd additions. The equilibration period ensured that equilibrium was reached for trace metal complexation by EDTA. If medium was prepared in advance, it was stored at 4°C. The experimental media were inoculated from stock cultures maintained in autoclaved Aquil medium. Cultures were acclimated in one transfer of metal-defined medium with the desired Cd′ and Zn′ levels prior to all experiments.

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* The total Cd was also an order of magnitude lower at 10 μM EDTA than at 100 μM EDTA for the same calculated Cd′, so the amount of ^{109}Cd added was also an order of magnitude lower.

Growth conditions

Light levels and concentrations of inorganic Zn, inorganic Cd, and total EDTA for cultures grown for each experiment are given in Table 2-1. Cultures for determining the effect of varying Cd′ on growth rate, cellular Cd quotas and Cd fractionation were grown in 30 mL, acid washed, polycarbonate tubes. Cultures for determining the effect of varying Zn′ on growth rate were also grown in 30 mL tubes. For all other experiments, cultures were grown in acid washed, polycarbonate 1 L square bottles or 3 L Fernbach flasks. Cultures were incubated at 21°C under constant light.

Cadmium quotas

As described above, cells were acclimated in one transfer of metal-defined medium prior to the experiment. An inoculum of acclimated cells was then added to ^{109}Cd radiolabelled medium with the same Zn′ and Cd′ levels. Cells were harvested in late exponential growth by filtering them under gentle vacuum (<5" Hg) onto a 3μm polycarbonate membrane filter. Metal associated with the cell surface was removed by incubating for 10 minutes in a 1 mM solution of diethylenetriaminepentaacetic acid (DTPA) in filtered seawater. Similar techniques have been developed to measure intracellular nickel (Price and Morel 1991) and iron (Hudson and Morel 1989). The sample was then washed 3 times with 5 mL filtered seawater. Care was taken throughout to prevent cell breakage by preventing the cells from drying during the washes and using less than 5" Hg vacuum. The activity of the cells on the filter was determined using a Beckman LS1801 liquid scintillation counter. The cell density was determined by counting cells stained with Lugol's solution in a hemocytometer. The Cd quota (the intracellular Cd concentration per cell) was calculated using the specific activity of the medium and the cell density at the time of measurement.

Cadmium fractionation

Cultures were grown and harvested as described above for Cd quotas. Cells were resuspended in 1000 µL filtered seawater, the filter was removed, and cells were frozen at -70°C. To determine partitioning between membrane-bound and soluble Cd in the cell, samples were thawed and ground for 4 min using a mechanically driven 2 mL Potter-Elvehjem tissue grinder at 4°C. Samples were centrifuged at 16,000 g in a Eppendorf 5415C centrifuge with a fixed angle rotor for 20 minutes at 4°C. The supernatant was decanted and the pellet resuspended in either 1000 µL filtered seawater or 1 N HCl and let stand overnight. The activity in the resuspended pellet and supernatant was then counted using a Beckman LS1801 liquid scintillation counter and corrected for a blank of approximately 50 cpm.

Cadmium uptake kinetics

Cultures were harvested in late exponential phase by filtration onto a polycarbonate membrane filter. Cells were resuspended in a small volume of 10 µM EDTA in chelexed synthetic ocean water without trace metals. Aliquots of the resuspended cells were added to synthetic ocean water that had been run through a chelex column (see Price et al. 1988/89) containing 10 µM EDTA and a range of radiolabelled Cd concentrations. Cadmium quotas were measured every half hour as described above. Cell density was estimated from fluorescence. During uptake, cells were maintained at a constant photon flux density of 30 $\mu E \cdot m^{-2} \cdot s^{-1}$.

The uptake rate at a given Cd′ concentration was determined from a linear regression of radiolabelled Cd taken up into the cells vs. time. Uptake rates were calculated from data collected during the first two hours following resuspension since uptake began to slow significantly over longer time periods. The effect of Zn on short term Cd uptake at 1800 pM Cd′ was determined by comparing uptake rates with no added Zn, 0.2 pM Zn′ and 16 pM Zn′. The kinetic parameters of uptake were determined by fitting uptake rates measured at 210, 410, 820, 1500, 3000, and 6000 pM Cd′ with the Michaelis-Menten equation.

Carbonic anhydrase (CA) assay

Cells were harvested when they reached at least half maximal cell density. They were resuspended in 10 mL filtered seawater, pelleted by centrifugation for 10 minutes at 1400 g in a Beckman TJ-6 centrifuge with a swinging bucket rotor, and frozen at –20°C. Cells were disrupted by sonication using a Branson Sonifier 250 sonicator with a microtip for 45 seconds at 80% duty cycle with output 5 while keeping the sample on ice. Unbroken cells and cellular debris were removed by centrifuging at 16,000 g in a Eppendorf 5415C centrifuge with a fixed angle rotor for 20 minutes at 4°C. The supernatant was decanted and an aliquot was assayed for total protein using the BCA protein reagent assay (Pierce). Samples were stored at –20°C.

Carbonic anhydrase activity was detected using a post-electrophoresis enzyme assay. Non-denaturing polyacrylamide gel electrophoresis was carried out according to the Laemmli method (Ausubel et al. 1992) on a 10% polyacrylamide gel. Carbonic anhydrase was detected by a modification of the method of Patterson et al. (1971). After electrophoresis, the gel was soaked in a solution of 0.1% bromcresol purple in fresh electrophoresis buffer. The gel was blotted dry and placed in a pure CO_2 atmosphere until red or yellow bands of CA activity appeared against the purple background. The gel was then frozen on dry ice and photographed with a Wratten 74 green filter under long wave UV light using Polaroid Instant black and white film.

Carbonic anhydrase activity of cells grown at different Cd′ and Zn′ levels was compared in samples containing 100μg of total protein. Samples were prepared and enzyme activity was assayed following non-denaturing gel electrophoresis as described above.

To compare the elution profiles of Cd and CA, carrier-free ^{109}Cd was added to cultures twenty hours before harvesting, during exponential growth. The radiolabel was not equilibrated with EDTA before addition to the medium, thus the concentration of Cd′ and the specific activity were not well defined after addition of the radiolabel. Cells were harvested, proteins extracted, and CA activity assayed as described above. The gel electrophoresis sample contained 80 μg total protein and 11 nCi of activity. A

Cd elution profile was determined by autoradiography of the dried gel using intensifying screens and a 1 month exposure.

Results

The ability of Cd to act as a nutrient for T. weissflogii depends on the concentrations of inorganic cadmium (Cd′) and zinc (Zn′) in the medium. We thus determined the optimal range of Zn′ concentrations at which Cd increases growth rates and the lowest Cd′ concentration that is effective. As described in the methods section, inorganic cobalt was eliminated from the culture medium in all experiments. Above a Zn′ concentration of 160 pM, cultures are able to grow at optimal rates and Cd does not affect growth rate (Figure 2-1). At limiting Zn′, 3 and 16 pM, adding Cd enhances growth rates of cultures by 30-100% (Figure 2-1). When no Zn was added to the growth medium, Zn contamination in this particular experiment was sufficient to allow cultures to grow, albeit slowly (Figure 2-1); in other growth experiments, however, T. weissflogii was not able to grow at all unless at least 2 pM Zn′ (total Zn concentration of 8 nM at 100 μM EDTA) was added. From Zn analyses of vitamin mixes added to all media (E. Boyle, pers. comm.), Zn contamination is estimated to be less than 0.1 nM, a level which would not support significant growth at 100 μM EDTA. Without any added Zn in the medium, adding 5 pM Cd′, which is beneficial at higher Zn′ levels, becomes lethal (Figure 2-1).

The Cd′ concentration used in Figure 2-1, 5 pM, is the minimum that significantly enhances growth of Zn-limited cells. This value was determined by adding varying amounts of Cd′ to cultures with only 3 pM Zn′. As shown in Figure 2-2 A, the lowest Cd′ level tested, 0.5 pM, does not significantly enhance growth of low Zn′ cells. Zinc-limited cells supplemented with 5 to 23 pM Cd′, however, grow almost twice as fast as those without added Cd. Above 23 pM, the toxic effects of Cd negate the beneficial effects shown at lower Cd levels and growth is not significantly faster than without added Cd. In contrast to the results obtained at 3 pM Zn′, at 16 pM Zn′ the beneficial effect of Cd is smaller but increases throughout the concentration range. As at 3 pM Zn′, increasing Cd at 16 pM Zn′ eventually elicits a toxic response (Ahner et

al. in press) but the toxicity threshold was not reached at the Cd′ values in Figure 2-2 A*.

In addition to growing faster with Cd, Zn-limited cultures for the most part also have higher intracellular Cd quotas (Figure 2-2 B). At the lowest Cd′ levels, 0.5 and 5 pM, low Zn′ cells have the same quotas as high Zn′ cells. Above 5 pM Cd′, quotas of low Zn′ cells are approximately 10 amol Cd·cell^{-1} higher than at high Zn′. As a fraction of the total, this difference is most dramatic at intermediate Cd′ levels (23 and 46 pM Cd′) where quotas of high Zn′ cells are half those of low Zn′ cells. Not only is the fractional difference smaller at 460 pM Cd′, but the variability in the quota measurements are of comparable magnitude to the difference.

The proportion of Cd which is membrane-bound and the proportion which is contained in the cytoplasm (an operational definition, see Methods) are roughly equal over a range of 0.5 pM to 460 pM Cd′ (Figure 2-2 C). At 4600 pM Cd′, however, only approximately 20% the total cellular Cd is membrane-bound. Because Cd fractionation was not affected by Zn, measurements at Zn′ concentrations of 16 pM and 3 pM were averaged in Figure 2-2 C.

To further characterize the relationship between Zn and Cd, we examined the effects of both Cd′ and Zn′ on short term Cd uptake rates. Short term Cd uptake rates are unchanged by the Zn′ concentration in the uptake medium (Figure 2-3); however the Zn′ concentration at which cells were acclimated does affect Cd uptake kinetics (Figure 2-4). The maximum uptake rate (ρ_{max}, Figure 2-4) depends on both the Zn′ and Cd′ levels at which cultures were acclimated†. Cells grown at 2 pM Zn′ without Cd have the highest value of ρ_{max}. Growing cells at 2 pM Zn′ with 460 pM Cd′ decreases

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* In Figure 2-2 A the maximum Cd′ was 460 pM.

† The Cd uptake data was fit by non-linear regression with the Michaelis-Menten equation: $\rho = \frac{Cd'}{Cd' + K_m}\rho_{max}$, where ρ is the Cd uptake rate, in amol Cd·cell^{-1}·min^{-1}; K_m is the half saturation constant; and ρ_{max} is the saturated Cd uptake rate, in amol Cd·cell^{-1}·min^{-1}.

ρ_{max} by 20%. Adding both 460 pM Cd′ and 16 pM Zn′ to the medium further lowers ρ_{max} by 30%. In contrast, within the resolution of our short term uptake data, the half saturation constant of the Cd transport system, K_m, is not affected by Cd and Zn acclimation. For all three cultures K_m is 2900 pM, six times higher than the highest Cd′ concentration at which cultures were grown.

The short-term and steady-state uptake rates at low Zn′ cannot be directly compared because different Zn′ concentrations were used. At high Zn′ and high Cd′, the steady-state uptake rate calculated from the product of the Cd quota and exponential growth rate is twice the short-term value. This difference is most likely due to cell stress during short-term uptake experiments or slowed growth at the end of the steady-state experiments when quotas were measured.

Supplementation of Zn-limited cells with only 5 pM Cd′ not only enhances growth but also substantially restores the activity of carbonic anhydrase (CA) in Zn-limited cells *in vivo*. The gel shown in Figure 2-5 compares the CA activity in extracts of cells grown at different Zn′ and Cd′ levels. No enzyme activity was detected in Zn-limited cultures supplied with only 0.5 pM Cd′ (C). However, increasing Cd′ to 5 pM (B) restores CA activity to levels comparable to those found in Zn-sufficient cells (A).

Restoration of CA activity by Cd could occur through either direct or indirect means. Since Cd coelutes with CA activity (Figure 2-6), it is likely that the increased activity is due to production of active Cd-CA. However the question of CA in *T. weissflogii* is more complicated than our earlier gels (Figure 2-5 and Figure 1 in Morel et al. 1994) would suggest. Improvement in the sensitivity of the technique led to consistent detection of multiple isoforms* of CA with an elution profile typical of that shown in Figure 2-6: a faint doublet labelled 1, another faint doublet labelled 2, and three broad bands of which only two can be distinguished in this particular gel (3 and 4). The Cd elution profile also shows distinct bands, the most intense of which coelutes with CA band 3. Two fainter bands of Cd activity also coelute with CA

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* Isoforms are proteins which have the same biochemical function but differing amino acid sequences.

doublet 2. No significant Cd activity appears to coelute with CA band 4, however, indicating Cd may not replace Zn in some CA isoforms.

Discussion

In Zn-limited *T. weissflogii* Cd clearly acts as a nutrient: very low inorganic Cd′ concentrations stimulate growth; the uptake of Cd and its intracellular quota are regulated over a range of Cd′ and Zn′ concentrations; and the biochemical role of Cd appears at least partly linked to carbonic anhydrase (CA) activity.

Like other micronutrients such as zinc (Sunda and Huntsman 1992) and copper (Manahan and Smith 1973), cadmium has a range of concentrations that are optimal for algal growth. At low levels of Cd′, growth of Zn-limited cells is slowed by metal deficiency. Unlike other micronutrients, however, Cd can apparently be completely replaced by another trace metal, Zn, if it is available in sufficient supply. Although *T. weissflogii* does not have an absolute requirement for Cd, we have found that it does have one for Zn. Below a Zn′ of 2 pM, *T. weissflogii* is unable to grow regardless of inorganic cadmium concentrations in the medium (Figure 2-1). The high growth rates of *T. weissflogii* with added Cd but without added Zn observed by Price and Morel (1990) were likely due to low levels of Zn contamination. Zinc and cadmium contamination also likely affect some of our results: growth rates at 10 μM EDTA have higher averages and greater variability than at 100 μM EDTA (for the same calculated Zn′ and Cd′ concentrations assuming no contamination). This is because even subnanomolar contamination of Zn or Cd is quite significant at the extremely low total Cd and Zn concentrations used at 10 μM EDTA (see Table 2-2).

The concentrations at which Cd partially replaces Zn in this study of *T. weissflogii* are close to the values of Cd′ and Zn′ measured by Bruland (1989, 1992) in the euphotic zone of the central North Pacific*. Although total Zn concentrations in surface ocean waters were 1 to 2 orders of magnitude higher than Cd (as is generally true in oligotrophic surface waters), Zn was found to be much more extensively complexed by strong organic chelators than Cd. In the surface waters (<100 m depth),

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* Earlier work on Cd/Zn replacement (Price and Morel 1990) used much higher Cd′ concentrations (460 pM) than those used here and measured in the surface ocean.

the estimated Cd′ concentration averaged ~1 pM and the estimated Zn′ concentration averaged ~6 pM. Between 100 m and 150 m (the bottom of the euphotic zone), Cd′ increased to 11 pM while the estimated Zn′ concentration remained constant at an average value of ~6 pM (data from Bruland 1989 and 1992, presented in Figure 2-7). If *T. weissflogii* lived near the bottom of the euphotic zone in the central North Pacific, the available Cd would enable it to overcome otherwise limiting Zn′ concentrations, assuming only the inorganic Cd and Zn were available.

Cobalt, which has also been shown to replace zinc in *T. weissflogii*, is present at levels in the surface ocean which are lower than Cd (Martin et al. 1989,1990). Like Zn and Cd, much of the inorganic cobalt in the open ocean may be organically complexed (and thus would not be bioavailable) judging from measurements of the speciation of Co in coastal waters and preliminary measurements in oligotrophic waters (Zhang et al. 1990, Donat and Bruland 1988).

Coastal algae such as *T. weissflogii* are susceptible to metal limitation at significantly higher concentrations than their oceanic cousins (Sunda and Huntsman 1992) and the range of Cd′ and Zn′ concentrations at which Cd can substitute for Zn in oceanic species may be lower than those for *T. weissflogii*. The extremely small size compared to eukaryotic phytoplankton of the numerically dominant picoplankton *Prochlorococcus* and *Synechococcus* makes it unlikely that they would be Zn-limited in the open ocean, and we do not expect Cd/Zn replacement to be important for these algae.

Cadmium might also be toxic to *T. weissflogii* at the concentrations measured in the open ocean if Zn′ is low enough (e.g. 0.2 pM Zn′, Figure 3-1 B). Certainly, the low concentrations of Cd′ which promote growth of moderately Zn-limited cultures* do not allow growth when no Zn is added (Figure 2-1). Although this effect might be due simply to lack of Zn contamination (assuming no Cd controls were contaminated with enough Zn to allow growth), a similar antagonistic effect is observed at higher Cd′

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* 3 pM Zn′

concentrations (>23 pM Cd′), where Zn-limited* but not Zn-sufficient† cells show a decrease in growth rate (Figure 2-2 A). At sufficient Zn, Cd′ levels must reach 4.6 nM before any deleterious effect on growth of *T. weissflogii* is observed (Ahner et al. in press). The net effect of Cd on growth reflects both beneficial and toxic interactions with the metabolism of the cell. The balance between these interactions depends not only on the level of Cd but also of Zn (and perhaps inorganic cobalt) available to the cell.

One of the important beneficial effects of Cd is the enhancement of the activity of the zinc metalloenzyme carbonic anhydrase (CA). Adding as little as 5 pM Cd′, the lowest level which enhances growth, restores CA activity to the same degree as the hundred times higher Cd′ levels‡ used in previous studies (Morel et al. 1994). This effect may in fact be the key to Cd/Zn replacement since Morel et al. (1994) have shown that a very large fraction of the total cellular Zn in *T. weissflogii* is associated with CA. Since so much of the cellular Zn is required for CA, Cd and Zn would likely have to be present at similar levels intracellularly for Cd to completely replace Zn in CA. Zinc quotas of 70 amol/cell measured previously for *T. weissflogii* (Price and Morel 1990) are somewhat higher than Cd quotas of 8-23 amol/cell that we measured in cells where Cd is replacing Zn further indicating that the substitution in CA is only partial.

Cadmium coelutes with only some of the multiple isoforms§ of CA produced by *T. weissflogii.* Given that Cd can substitute for Zn in mammalian CA and still retain significant activity (Bertini et al. 1987; Bauer et al. 1976; Bertini and Luchinat 1983), it is plausible that these Cd-substituted isoforms of CA in *T. weissflogii* should be active. Other species of microalgae produce multiple isoforms of CA which are associated variously with the chloroplast, the cytoplasm and cell membranes (Williams and

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* 3 pM Zn′

† 16 pM Zn′

‡ This higher Cd′ level was used in our previous study because it was the same Cd′ concentration which restored growth in Price and Morel (1990).

§ Isoforms are proteins which have the same biochemical function but differing amino acid sequences.

Coleman 1993; Goyal et al. 1992; Fukazawa et al. 1991; Husic et al. 1989). Some forms of CA are thought to be involved in bicarbonate uptake (Sultemeyer et al. 1989), thus cadmium/zinc replacement in CA may facilitate adaptation to low PCO_2 under Zn-limited conditions. We are currently studying the differences in the physiological role and metal content of the CA isoforms of *T. weissflogii.*

Because cadmium acts as a nutrient, as shown by its effects on growth and enzyme activity, it is not surprising that the uptake of cadmium, like other nutrients, is regulated in such a way as to maintain relatively constant intracellular concentrations. The inverse relationship between ρ_{max} and nutrient concentration in the growth medium observed for iron (Harrison and Morel 1986), manganese (Sunda and Huntsman 1985) and zinc (Sunda and Huntsman 1992) also holds for cadmium. Through increases in ρ_{max} the intracellular levels of nutrients are maintained at optimal levels as external concentrations decrease. Decreasing K_m at low nutrient concentrations could have the same result, but as for other micronutrients such as Fe and Mn, the K_m for Cd remains constant as metal concentrations in the growth medium vary. It seems that the number of uptake ligands, not their substrate affinity, is under feedback control. Since the values of K_m are much higher than ambient metal levels, it appears to be most efficient for the transport system of Fe, Mn, and Cd to operate far from saturation as has been argued by Hudson and Morel (1993).

Zinc as well as cadmium plays a role in the regulation of Cd transport. Increasing Zn′ in the growth medium reduces ρ_{max} for Cd uptake (Figure 2-4). In contrast, over the short term uptake of Cd does not appear to be affected by Zn (Figure 2-3), indicating perhaps that Cd is not taken up by the same transport protein as Zn. In view of the large K_m, however, much higher Zn′ levels than 16 pM might be required to see a competitive effect.

In general, Cd quotas vary with inorganic Zn availability. Above 5 pM Cd′, low Zn′ cells have significantly higher Cd quotas than high Zn′ cells. Other studies have also found that Cd quotas were higher in Zn-limited than in Zn-sufficient *T. weissflogii* cells, although the Cd quotas they measured were somewhat lower than those

measured here perhaps due to Zn contamination, Cd contamination, or the presence of inorganic Co in the medium in their studies (Price and Morel 1990, Ahner and Morel in press). In contrast, at lower Cd′ concentrations there is no change in quotas with varying Zn′. This was unexpected since low Zn′ cultures were growing more slowly than those at high Zn′ so that even if uptake were diffusion limited, as it is likely to be at these Cd′ levels (Hudson and Morel 1993), low Zn′ cells should have had somewhat higher Cd quotas.

The regulation of Cd transport and quotas in *T. weissflogii* mirrors the bioregulation of Cd implied by the correlation of cadmium with phosphate observed in ocean waters. Assuming a constant cellular phosphate quota of 0.1 pmol for *T. weissflogii* (Price and Morel 1990), we calculate intracellular Cd:PO_4 ratios of 0.1×10^{-4} to 4×10^{-4}. These values agree well with particulate Cd:PO_4 values ranging from 1-10×10^{-4} measured in ocean samples (Sherell 1989). At low Cd′ (pCd>10), the Cd quotas in *T. weissflogii* fall into the mid range of the values measured by Ahner et al. (in press) which show a high degree of interspecies variation at a given Cd′, but no clear pattern of differences between coastal and oceanic species. Thus the coastal diatom *T. weissflogii* may be a reasonable model for the biological cycling of Cd in the open ocean.

At high Cd*, there is a shift in Cd from membrane-bound material to the cytoplasm, perhaps due to the production of the soluble Cd-phytochelatin complex (Ahner and Morel in press) as a detoxification mechanism. Cadmium fractionation is fairly constant otherwise over the range of external Cd′ concentrations that are relevant to the open ocean. The fractionation of Cd between the cell cytoplasm and cell membrane is important because the fraction of a given element assimilated by copepods during grazing as compared to the amount packaged into fecal material has been shown to be directly proportional to the distribution of that element between the cytoplasm and cell membranes in their algal food (Reinfelder and Fisher 1991). The value of 40%

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* 4.6 nM Cd′

for the fractionation of Cd measured here, which is consistent with previous measurements of 60% (Reinfelder and Fisher 1991; Price and Morel 1990), is also very similar to that the value of 40% for PO_4 (Reinfelder and Fisher 1991). As a result, the same Cd:PO_4 ratio found in phytoplankton is likely to be preserved in fecal pellets, some fraction of which are exported to the deep ocean and remineralized. Through this mechanism, the ratio of concentrations of cadmium and phosphate dissolved in the ocean may simply reflect the average composition of phytoplankton. Thus the similar vertical profiles of Cd and PO_4 in the ocean likely reflect their respective roles as algal nutrients and the similarities in their particulate to soluble distributions in phytoplankton.

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Figure 2-1. Effect of varying Zn′ on growth rate in the presence and absence of Cd.

Growth rates of *T. weissflogii* with 5 pM Cd′ (■) and no added Cd′ (□) are plotted vs. the concentration of inorganic Zn (Zn′) added to the medium. Cultures were grown at a constant photon flux density of 120-130 $\mu E \cdot m^{-2} \cdot s^{-1}$. Data are averages of three replicate cultures with 1 standard deviation error bars.

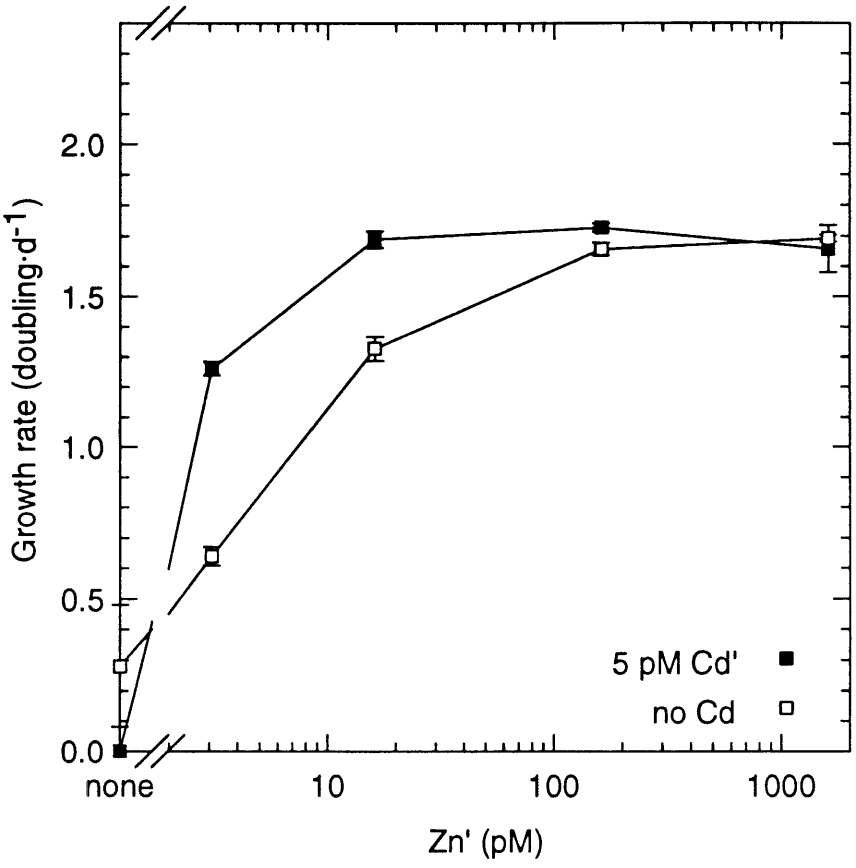
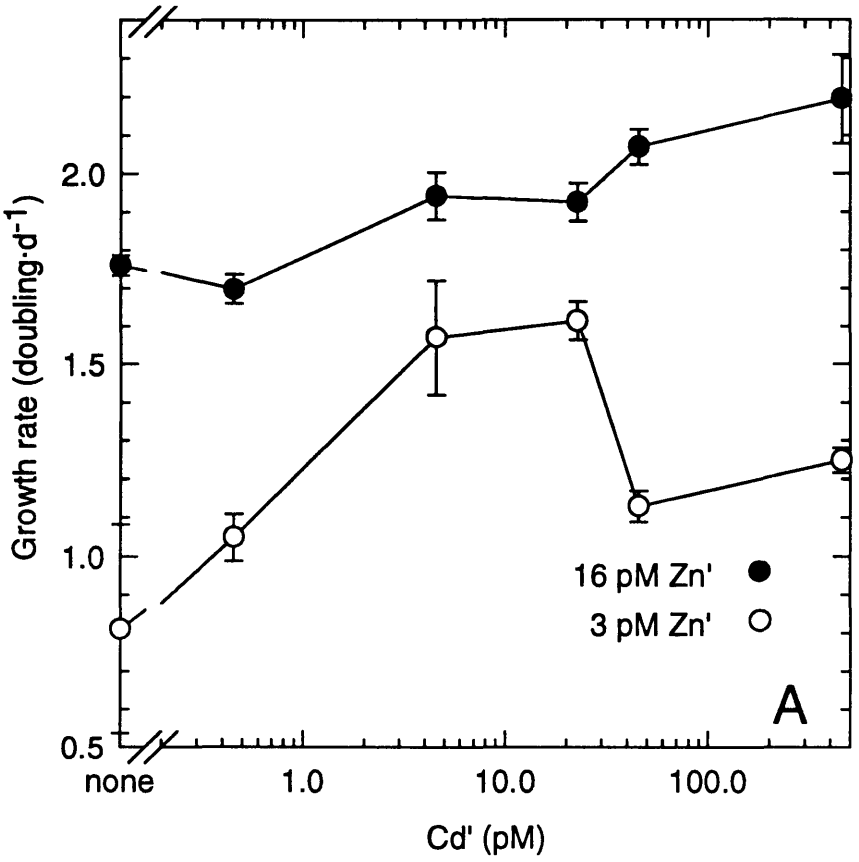
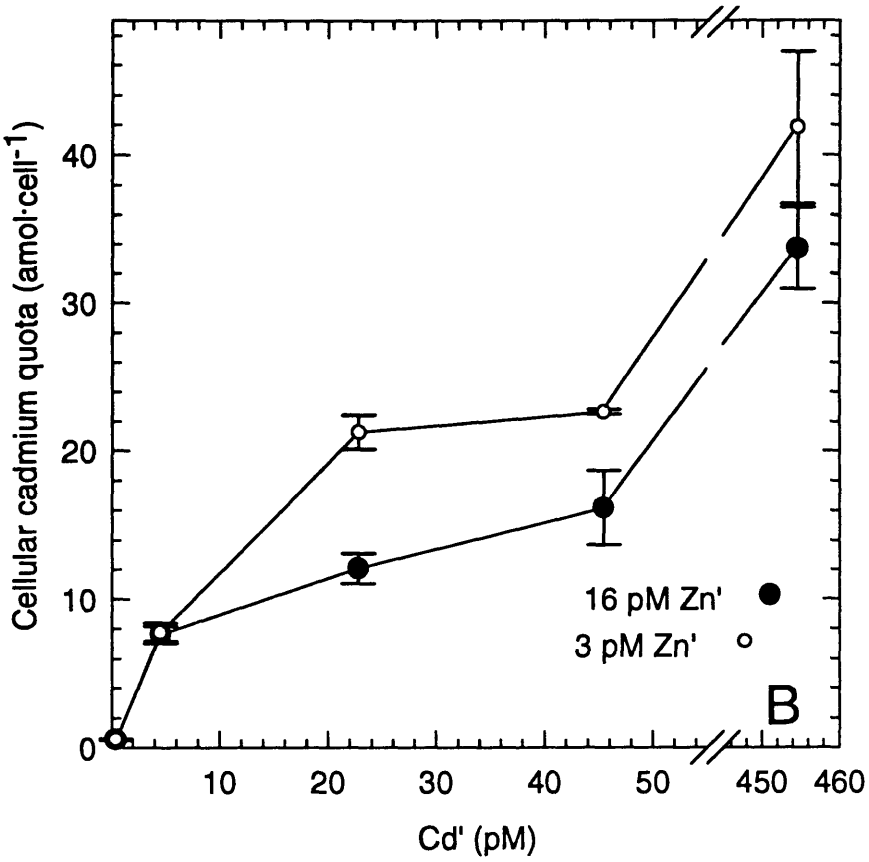

Growth rate (doubling·d-1)
2.0
1.5
1.0
0.5
0.0
none
10
100
1000
Zn' (pM)
5 pM Cd'
no Cd

Figure 2-2. Effect of varying Cd′ on growth rate, Cd quotas, and cellular Cd fractionation at high and low Zn′.

Growth rates (A) and intracellular Cd quotas (B) of *T. weissflogii* at 16 pM Zn′ (●) and 3 pM Zn′ (○) are plotted vs. the concentration of inorganic Cd (Cd′) in the medium. In panel C, membrane bound Cd as a percent of total intracellular Cd is plotted vs. Cd′. Cultures were grown at a constant photon flux density of 90-100 $\mu E \cdot m^{-2} \cdot s^{-1}$. Data in panels A and B are averages of three replicate cultures (except for cultures to which no Cd was added, which are averages of 12 replicate cultures). Data in panel C are averages of 3 replicates at 3 pM Zn′ and 3 at 16 pM Zn′. Error bars span one standard deviation above and below the mean.


Growth rate (doubling·d-1)
2.0
1.5
1.0
0.5
none
1.0
10.0
100.0
Cd' (pM)
16 pM Zn'
3 pM Zn'
A


Cellular cadmium quota (amol·cell^{-1})
0
10
20
30
40
10
20
30
40
50
450
460
Cd' (pM)
16 pM Zn'
3 pM Zn'
B

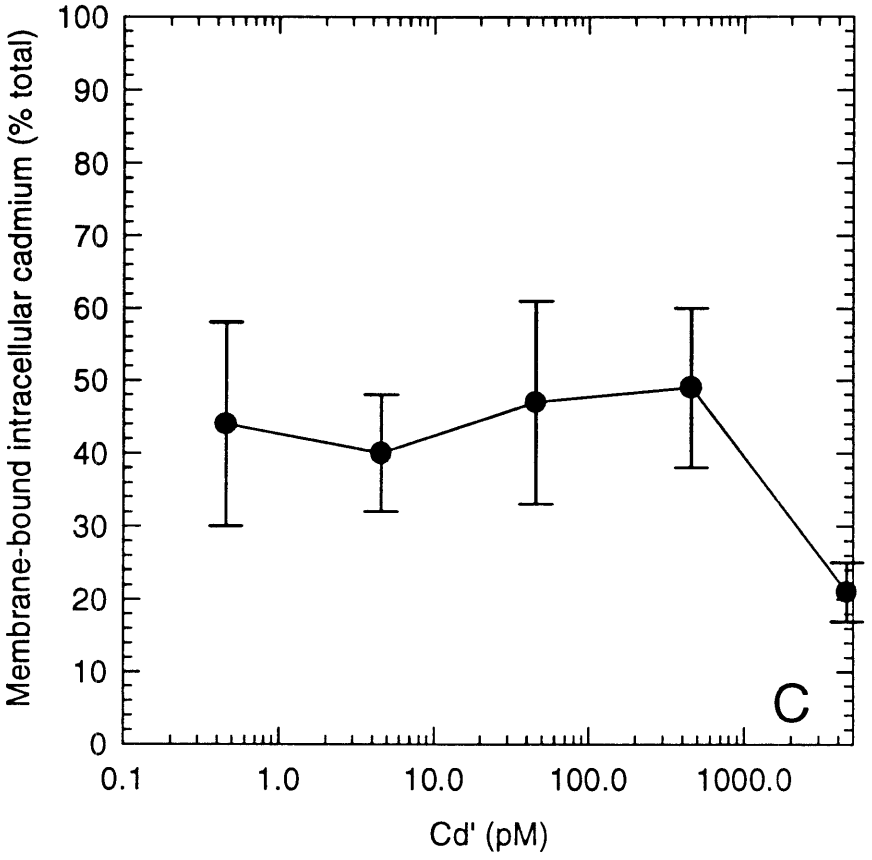

Membrane-bound intracellular cadmium (% total)
100
90
80
70
60
50
40
30
20
10
0
0.1
1.0
10.0
100.0
1000.0
Cd' (pM)
C

Figure 2-3. Short term Cd uptake rates at varying Zn′.

Intracellular Cd quotas are plotted vs. the length of time cells were exposed to radiolabelled Cd. *T. weissflogii* was grown in cold medium containing 460 pM Cd′ and 2 pM Zn′. In late exponential phase, cells were harvested and resuspended in medium containing 1800 pM radiolabelled Cd′ and varying amounts of Zn′: no added Zn (○), 0.2 pM Zn′ (●), and 16 pM Zn′ (■).

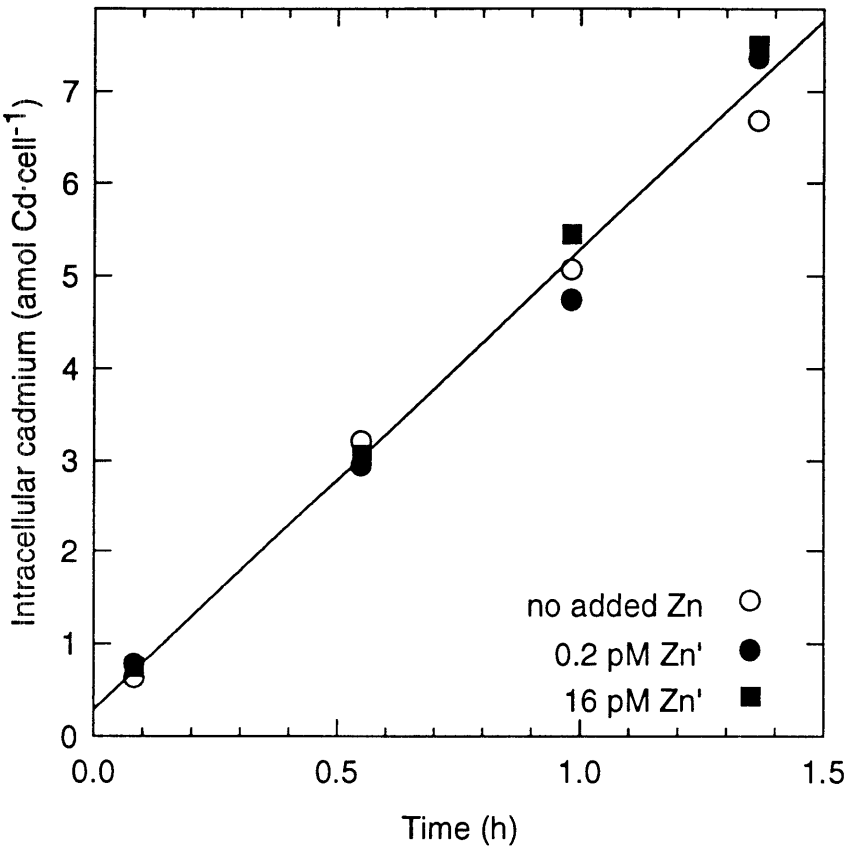

Intracellular cadmium (amol Cd·cell^{-1})
0
1
2
3
4
5
6
7
0.0
0.5
1.0
1.5
Time (h)
no added Zn
0.2 pM Zn'
16 pM Zn'

Figure 2-4. Kinetics of Cd uptake in cells acclimated at varying Cd′ and Zn′.

Short term Cd uptake rates are plotted vs. inorganic Cd concentration (Cd′) in the uptake medium. Cultures were grown in unlabelled medium containing 2 pM Zn′ without added Cd (□), 2 pM Zn′ with 460 pM Cd′ (■), and 16 pM Zn′ with 460 pM Cd′ (●). Cells were resuspended in medium containing varying amounts of radiolabelled Cd′. Uptake rates were determined as in Figure 2-3. Uptake data were fit by the Michaelis-Menten equation.

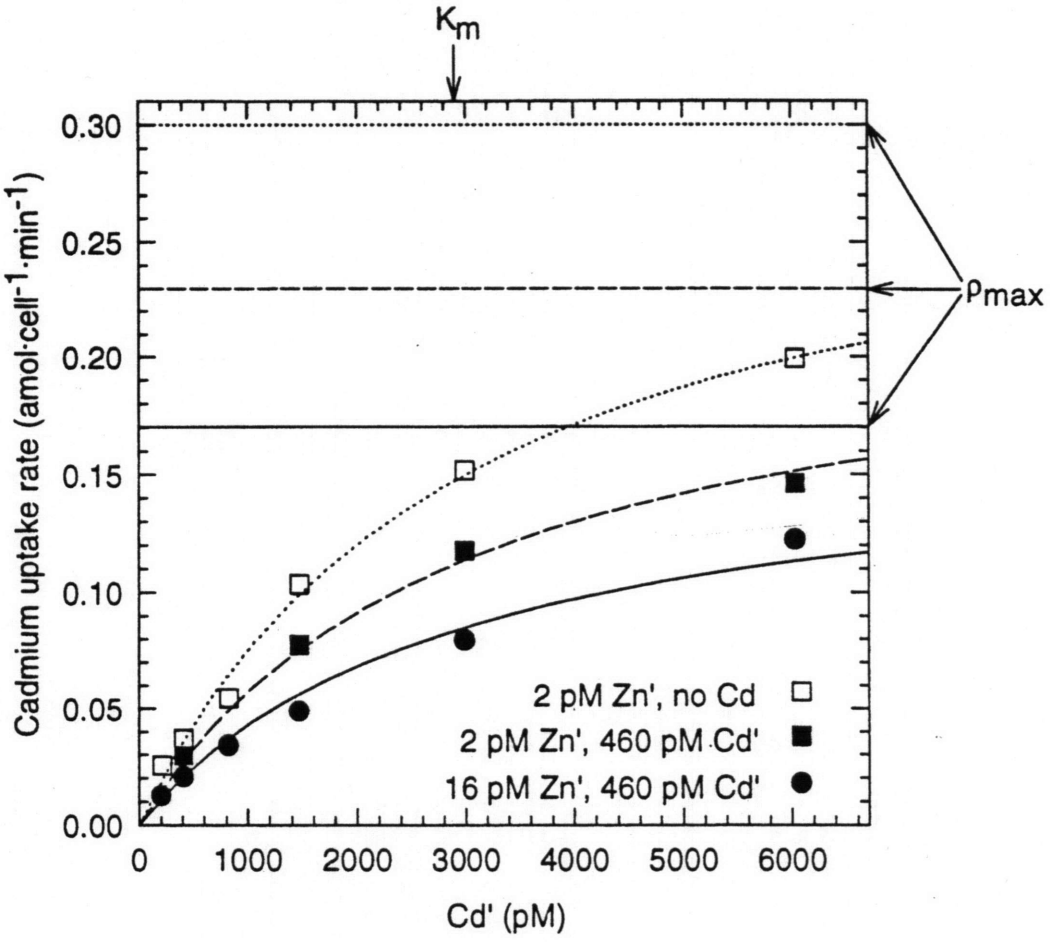

K_m
ρ_{max}
Cadmium uptake rate (amol·cell^{-1}·min^{-1})
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0
1000
2000
3000
4000
5000
6000
Cd' (pM)
2 pM Zn', no Cd
2 pM Zn', 460 pM Cd'
16 pM Zn', 460 pM Cd'

Figure 2-5. Effect of Cd/Zn replacement on carbonic anhydrase activity.

Cell extracts were prepared from cells grown at 16 pM Zn′ with 0.5 pM Cd′ (**A**), 3 pM Zn′ with 5 pM Cd′ (**B**), and 3 pM Zn′ with 0.5 pM Cd′ (**C**). Carbonic anhydrase activity was detected following non-denaturing electrophoresis using the fluorescent pH indicator bromcresol purple to show the pH change coincident with the hydration of CO_2.

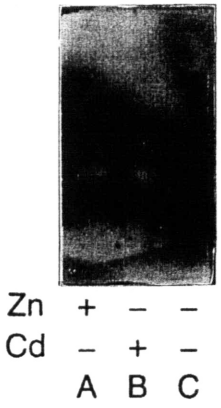


Figure 2-6. Comparison of Cd and carbonic anhydrase elution profiles.

Carbonic anhydrase activity (**A**) and radiolabelled Cd (**B**) were assayed following non-denaturing gel electrophoresis of cellular extracts prepared from *T. weissflogii* grown at 16 pM Zn′ with 5 pM radiolabelled Cd′. Enzyme activity was assayed as described in Figure 2-5. Following the enzyme assay, ^{109}Cd was detected in the dried gel by autoradiography.

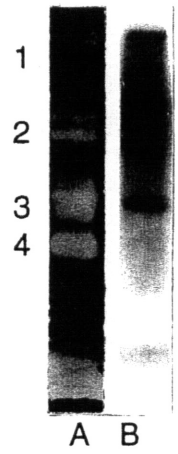


Figure 2-7. Cd′ and Zn′ in the central North Pacific.

Data from Bruland (1989, 1992) are presented on the variation of inorganic Cd (O, Cd′) and inorganic (●, Zn′) with depth for a station in the central North Pacific (Vertex IV, ~28°N, 150°W). These profiles reflect variation in both the total metal concentrations and the concentration of organic ligands which strongly complexed Cd and Zn. For illustration, the data were fit by a function whose value was the average Cd′ (dashed line) or Zn′ (solid line) concentration at depths of ≤100 m for Cd and ≤200 m for Zn (where the inorganic concentration of each of these metals was estimated rather than measured). Below these depths, the data were fit by a third order polynomial. The depth of the euphotic zone was 150 m and the depth of the mixed layer was 25 m at this station.

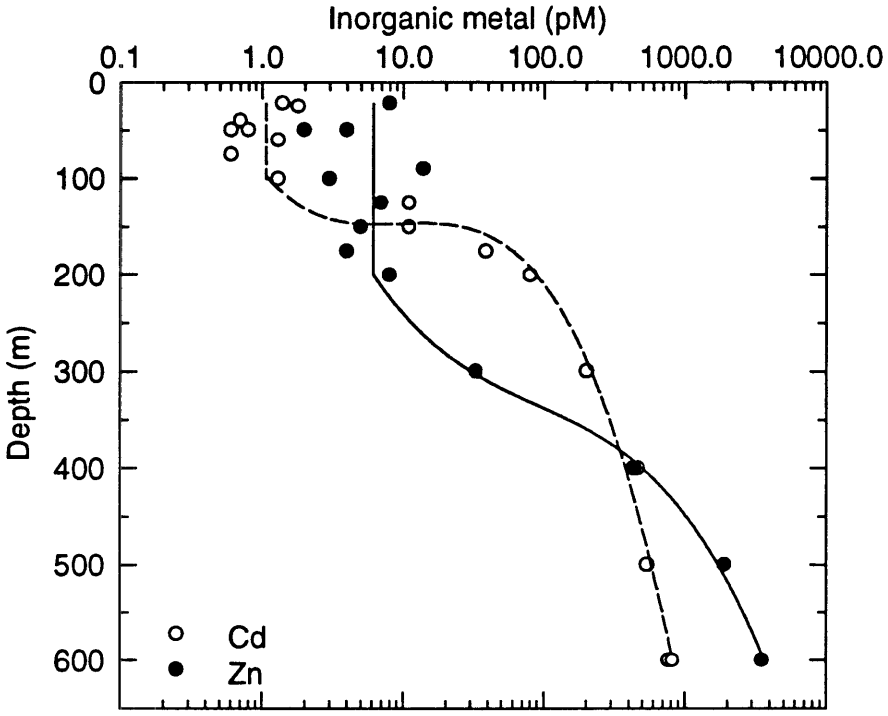

Inorganic metal (pM)
0.1
1.0
10.0
100.0
1000.0
10000.0
0
100
200
300
400
500
600
Depth (m)
Cd
Zn

Table 2-1. Growth conditions of experiments.

Inorganic Zn and Cd concentrations (Zn′ and Cd′) were calculated using MINEQL (Westall et al. 1976). Light levels were measured using a QSL-100 Irradiance Meter (Biospherical Instruments).

Experiment	*Zn′* (*pM*)	*Cd′* (*pM*)	*EDTA* (*μM*)	*Light* ($\mu E \cdot m^{-2} \cdot s^{-1}$)
Growth at varying Zn′	none-1600	none, 5	100	120-130
Growth and quotas at varying Cd′	3, 16	none-460	10	90-100
Cellular fractionation at varying Cd′	3, 16	5-4600	10	90-100
	"	0.5	100	"
Cd uptake at varying Zn′	2	460	100	160-170
Cadmium transport kinetics	2, 16	460	100	160-170
	2	none	"	"
CA at varying Zn′ and Cd′	16	0.5	100	160-170
	3	0.5, 5	"	"
Cd and CA coelution	16	5	10	160-170

Table 2-2. Growth rates at high and low EDTA.

Inorganic cadmium and zinc concentrations (Cd′ and Zn′) were calculated using MINEQL (Westall et al. 1976). Growth rates are mean values (± 1 S.D.) determined during exponential growth.

Cd′ (pM)	*Zn′ (pM)*	*100 μM EDTA (doub./d)*	*10 μM EDTA (doub./d)*
none	3*	0.64±.03	0.8±.3
none	16†	1.33±.04	1.76±.03
5‡	3*	1.26±.02	1.6±.2
5‡	16†	1.69±.03	1.94±.06

* Total Zn = 16 nM with 100 mM EDTA and 1.6 nM with 10 mM EDTA
† Total Zn = 80 nM with 100 mM EDTA and 8.0 nM with 10 mM EDTA
‡Total Cd = 4.4 nM with 100 mM EDTA and 0.4 nM with 10 mM EDTA

Chapter 3 Replacement of Zinc by Cadmium in Marine Phytoplankton*

Abstract

The concentration of cadmium varies like that of a nutrient in the open ocean. Detailed studies of the marine diatom *Thalassiosira weissflogii* have shown that cadmium can act as an algal nutrient under conditions of zinc limitation. We show here that cadmium can also enhance the growth of a variety of species, including a chlorophyte and some prymnesiophytes, when they are zinc-limited. The replacement of zinc by cadmium occurs at environmentally relevant inorganic cadmium and zinc concentrations. Very low concentrations of inorganic cadmium that are beneficial under conditions of moderate zinc-limitation become toxic in cultures severely limited by zinc. The role of cadmium as an algal nutrient is thus observable in a narrow, species-specific range of inorganic zinc and cadmium concentrations.

*This chapter is a modified version of Lee, J. G. and F. M. M. Morel. In press. Replacement of Zinc by Cadmium in Marine Phytoplankton. *Mar. Ecol. Prog. Ser.*

Introduction

The distribution of Cd within the ocean strongly suggests that it is used as a nutrient by marine phytoplankton. Like the major nutrients nitrate and phosphate, Cd is depleted from surface waters, increases with depth, and reaches a fairly constant concentration in deep waters (Boyle et al. 1976, Bruland & Franks 1983). This distribution is *prima facie* evidence of biological control over the distribution of Cd in the marine environment due both to remineralization (Lee & Fisher 1993) and scavenging in surface waters.

Until recently, however, Cd had no known role as a nutrient and rather was considered one of the most toxic trace metals. A few years ago, Price and Morel (1990) demonstrated that under conditions of Zn limitation, Cd restored the growth rate of the marine diatom *Thalassiosira weissflogii* to near maximal levels. In further work we have shown (Lee et al. in press) that Cd enhances the growth of Zn-limited cultures of *T. weissflogii* at inorganic Cd (Cd′) and Zn (Zn′) levels which are close to those that have been measured in the surface water of the open ocean (~6 pM Zn′, ~1 pM Cd′; Bruland 1989, 1992) although Cd cannot completely replace Zn. Over a wide range of external Cd′ and Zn′ concentrations, Cd uptake kinetics are regulated and the intracellular Cd quotas are maintained at relatively constant levels. The same low level of inorganic Cd that enhances the growth rate of Zn-limited cells restores the activity of carbonic anhydrase (Lee et al. in press), which may be the key enzyme limiting growth of *T. weissflogii* at low Zn′ (Morel et al. 1994). Cadmium also coelutes with some of the isoforms of carbonic anhydrase detected by a post-electrophoresis enzyme assay indicating that Cd substitution in carbonic anhydrase is likely partly responsible for the nutritional role of Cd.

If the nutrient role of Cd demonstrated for *Thalassiosira weissflogii* is the explanation for the nutrient-like distribution in the oceans, many other phytoplankton should be able to replace Zn with Cd. We have thus extended our detailed work with *T. weissflogii* to determine whether Cd can replace Zn in other species of marine

phytoplankton: a smaller coastal diatom and an oceanic diatom, two coastal and one oceanic prymnesiophyte, two chlorophytes, and a dinoflagellate.

Methods

Cultures of the nine species of marine phytoplankton listed in Table 3-1 (obtained from the Center for the Culture of Marine Phytoplankton, Bigelow Laboratory) were grown in synthetic ocean water prepared according to the recipe for Aquil (Price et al. 1988/89) with the following modifications: inorganic Co was omitted from the recipe since Co, like Cd, can substitute for Zn (Price & Morel 1990); Zn varied (inorganic Zn, Zn′, was reduced to 3 and 0.2 pM instead of the normal Aquil level of 16 pM); and Cd was added at a level of 5 pM inorganic Cd (Cd′). Inorganic trace metal concentrations, M′, were calculated from total concentrations using the computer program MINEQL (Westall et al. 1976). Details of media preparation are described in Lee et al. (in press). Maximal growth rates under Zn- and Co-sufficient conditions are also given in Table 3-1.

This metal-defined medium was inoculated from stock cultures maintained in autoclaved, metal-sufficient Aquil medium. Cultures were acclimated in one transfer of metal-defined medium with the desired Cd′ and Zn′ levels prior to all experiments. Cultures were grown in 30 mL, acid-washed, polycarbonate tubes. Cultures were incubated at 21°C under a constant photon flux density of 90-100 $\mu E \cdot m^{-2} \cdot s^{-1}$. Growth was monitored by in vivo fluorescence and growth rates were determined during exponential phase growth as per the method of Brand et al. (1981). Replicates were performed by subsequent transfer into fresh medium if Cd increased growth.

Results

We were able to lower Zn′ enough to limit growth for almost all of the species of algae we studied. The growth rates of *Thalassiosira pseudonana*, *Thalassiosira weissflogii*, *Pavlova lutheri*, *Pleurochrysis carterae*, *Tetraselmis maculata* and *Heterocapsa pygmaea* were less than maximal at a Zn′ level of 3 pM (Figure 3-1 A). *Emiliana huxleyi*, although not limited at 3 pM Zn′, was limited by Zn at 0.2 pM Zn′ (Figure 3-1 B). *Thalassiosira oceanica* and *Dunaliella tertiolecta*, however, were able to grow at near-maximal rates (99 and 82% respectively of maximum) even at 0.2 pM Zn′ (Figure 3-1 B).

At 3 pM Zn′, Cd acted as a nutrient for half of the species that were Zn-limited (Figure 3-1 A and Figure 3-2 A). Adding Cd had a significant beneficial effect on growth at the 95% confidence level for *Thalassiosira weissflogii*, *Pleurochrysis carterae* and *Tetraselmis maculata* (for at least one of the replicates). Cadmium may also have had a beneficial effect on the growth *of Thalassiosira pseudonana* and *Thalassiosira oceanica*, but this effect is not significant at the 95% confidence level for either replicate (and *T. oceanica* was not Zn-limited at 3 pM Zn′). Therefore a total of 3 out of the 6 species that were Zn-limited at 3 pM Zn′ were able to grow faster when supplied with Cd′ at very low concentrations (Cd′ = 5 pM, Figure 3-2 A). The growth curves for these species and *T. oceanica* with and without Cd are compared in Figure 3-2 A. At lower Zn′ concentrations of only 0.2 pM, Cd benefited a smaller fraction of the species limited by Zn: only *T. maculata* and *Emiliana huxleyi* grew significantly faster at the 95% confidence level when supplemented with Cd (2 out of 7 Zn-limited species, Figure 3-2 B). Reproducibility of growth rates of replicates was generally good (<10 % difference, Figure 3-1) except for *P. carterae* at 3 pM Zn′ without Cd and *T. maculata* at 0.2 pM Zn′ without Cd (12% difference for *P. carterae* and 39% for *T. maculata*) most likely due to Zn contamination of the second replicate during transfer.

At low concentrations of Zn′ (0.2 pM), Cd was toxic to at least five out of the nine species studied: *Thalassiosira oceanica*, *Dunaliella tertiolecta*, *Thalassiosira pseudonana*, *Thalassiosira weissflogii*, and *Pavlova lutheri* (Figure 3-1 B). The effects of Zn limitation and Cd toxicity could not be distinguished *for Pleurochrysis carterae* since cultures were not able to grow at all even in the absence of Cd at 0.2 pM Zn′. The same was true for *Heterocapsa pygmaea.* At the higher Zn′ concentration of 3 pM, Cd toxicity was only observed for one species, *H. pygmaea.*

Discussion

Our previous work on the replacement of Cd by Zn in *Thalassiosira weissflogii* has shown that limitation by Zn is a necessary condition for Cd to enhance the growth rate of cultures (Lee et al. in press). However, from our present study there does not appear to be a straightforward pattern for predicting which species are the most prone to Zn limitation. Small size does not appear to make Zn limitation less likely: *Thalassiosira pseudonana*, with a cell volume of only 71 μm^3, and *Pavlova lutheri*, with a cell volume of 75 μm^3 (see Table 3-1), were easily limited at 3 pM Zn′. Conversely, the larger alga *Dunaliella tertiolecta* (350 μm^3) was able to grow as fast at 0.2 pM as at 3 pM Zn′. Physiological differences which allow variations in the cellular Zn requirement must overcome physical limitations imposed by diffusion which would otherwise predict that large cells are more easily nutrient limited than small ones (Hudson & Morel 1993).

Neither do oceanic algal species always appear to be less subject to Zn limitation than coastal species. The oceanic species *Emiliana huxleyi* was Zn-limited at 0.2 pM Zn′ while the coastal species *Dunaliella tertiolecta* grew at maximum rates. Previous work has also shown that although coastal species are usually more easily limited by low Zn′ than oceanic species, that is not always the case (Brand et al. 1983). Cobalt limitation appears to play a synergistic role in Zn limitation for the oceanic coccolithophorid *E. huxleyi* since the same Zn′ levels found to be limiting in this study were not limiting when Co was provided (Sunda & Huntsman 1992).

For all species studied, the effect of Cd on growth rate, positive or negative, is highly dependent on the Zn′ concentrations in the medium (as well of course as the Cd ' concentration (Lee et al. in press)). For each species we can thus expect that the beneficial role of Cd will only be observed over a narrow range of Cd′ and Zn′ concentrations. In view of this difficulty, it is remarkable that we obtained positive results with so many species. The same low levels of Zn′ and Cd′ which resulted in a beneficial effect of Cd on *Thalassiosira weissflogii* also lead to an enhancement of

growth by Cd in several of the other species surveyed. This coincidence, which cuts across algal phyla, may be related to the fact that the Cd′ and Zn′ levels at which Cd replaces Zn in *T. weissflogii* are close to those which have been measured in the marine environment (Bruland 1989, 1992), although in an oligotrophic rather than coastal regime. It is of course entirely possible that other combinations of Cd′ and Zn′ concentrations than those used here might allow Cd/Zn replacement in the species which showed negative results.

As is the case for Cd/Zn replacement, the dependence of Cd toxicity on the inorganic Zn concentration in the medium gives a clue as to its mechanism. Either competitive inhibition of Zn and Cd uptake or biochemically ineffective replacement of Zn by Cd in proteins is the likely cause. In *Thalassiosira weissflogii*, competitive inhibition of Cd uptake by Zn does not appear important at the Zn′ and Cd′ levels studied here (Lee et al. in press). More likely, then, Cd toxicity is the result of the inactivation of Zn enzymes by Cd/Zn substitution under conditions of severe Zn limitation.

Algae and higher plants produce a metal-binding polypeptide, phytochelatin, in response to exposure to Cd and other trace metals (Grill et al. 1985; Gekeler et al. 1988). This response to Cd under controlled trace metal conditions has been studied in detail by Ahner et al. (in press) for all but one of the species examined here (*Thalassiosira pseudonana*). Exposure to Cd causes a much stronger response by *Thalassiosira weissflogii* in the cellular production of phytochelatin than any other trace metal (Ahner & Morel in press). We would therefore expect that phytochelatin production would play an important role in the ability of Cd to replace Zn. It is thus of interest to note that two of the species that have the greatest ability to increase the levels of phytochelatin/g chl a as Cd′ increases, *T. weissflogii* and *Tetraselmis maculata* (Ahner et al. in press), are also the species that show the clearest evidence of Cd/Zn replacement (Figure 3-2). The least response of phytochelatin/g chl a to Cd′ is that of *Heterocapsa pygmaea* (Ahner et al. in press) and this species is the most sensitive to Cd toxicity of those surveyed. The metal buffering capacity provided by phytochelatin

may therefore be important for Cd/Zn replacement, but there are some species differences in this response.

Acknowledgments. This research was supported by a grant to J. G. L. and F. M. M. M. from the Department of Defense NDSEG fellowship program, the National Science Foundation, the Office of Naval Research, and the Environmental Protection Agency.

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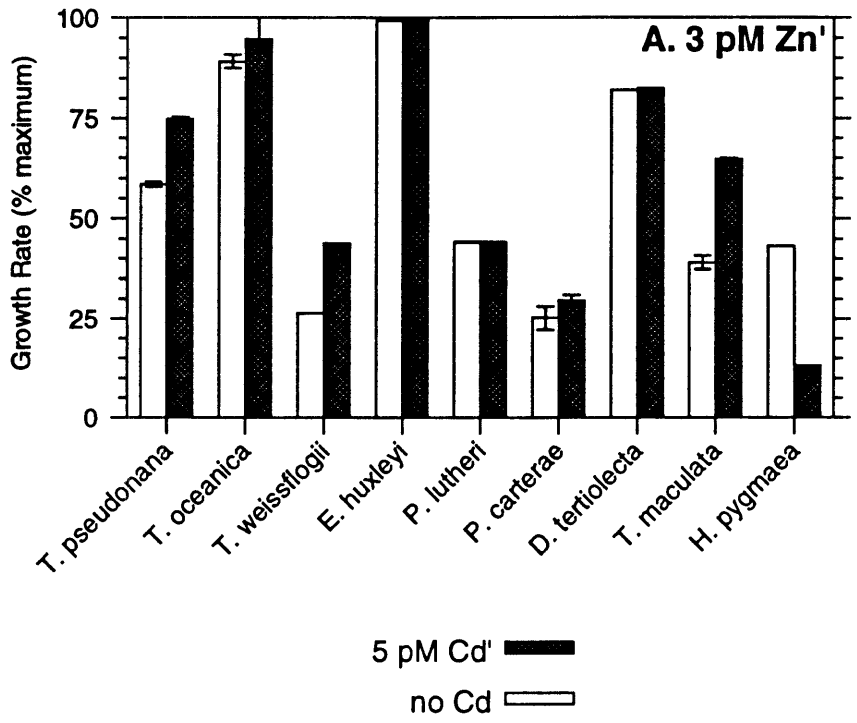
Figure 3-1. Effect of Cd on growth of marine phytoplankton at low inorganic Zn.

Inorganic Zn′ concentrations used were mildly limiting (**A**, 3 pM Zn′) and severely limiting (**B**, 0.2 pM Zn′) to *Thalassiosira weissflogii*. Filled bars represent cultures supplemented with 5 pM Cd′ and open bars represent no added Cd. Error bars span the range of duplicates. Cultures were pre-conditioned at a given Cd′ and Zn′ for one transfer. Growth rates were determined by monitoring in vivo fluorescence during exponential phase growth in subsequent transfers and normalized to maximum rates (see Table 3-1).


A. 3 pM Zn'
Growth Rate (% maximum)
100
75
50
25
0
T. pseudonana
T. oceanica
T. weissflogii
E. huxleyi
P. lutheri
P. carterae
D. tertiolecta
T. maculata
H. pygmaea
5 pM Cd'
no Cd

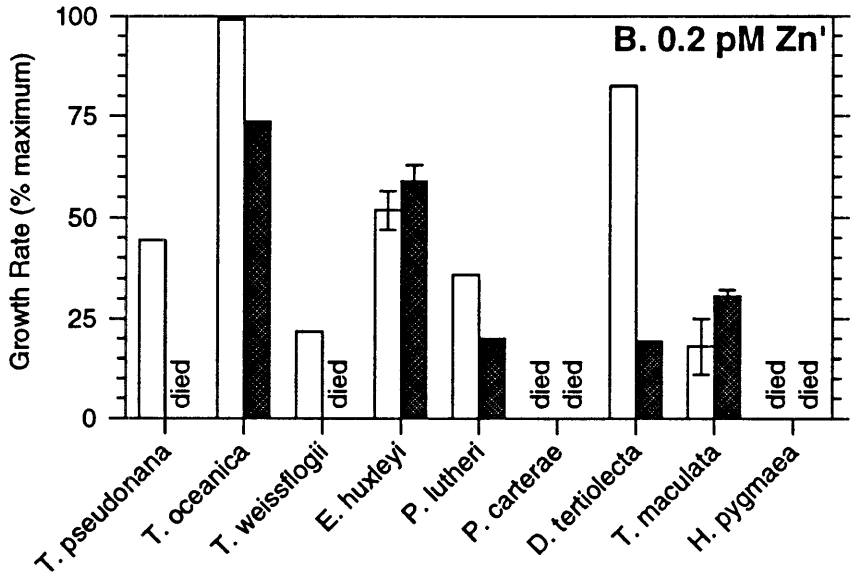
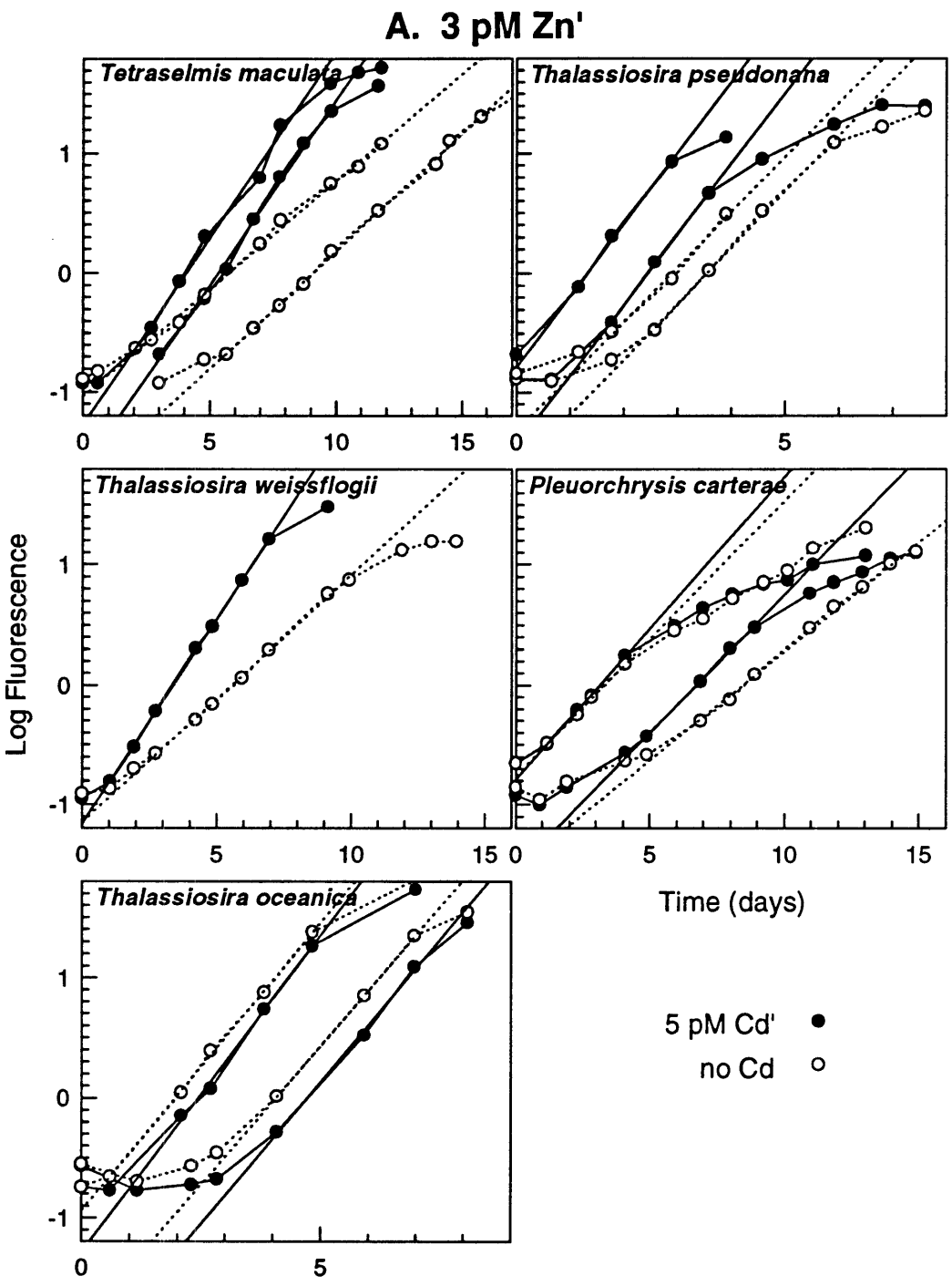

B. 0.2 pM Zn'
Growth Rate (% maximum)
100
75
50
25
0
died
T. pseudonana
T. oceanica
T. weissflogii
E. huxleyi
P. lutheri
P. carterae
D. tertiolecta
T. maculata
H. pygmaea

Figure 3-2. Growth curves of cultures where Cd had a beneficial effect.

The Zn′ levels in the media were 3 pM in **A** (mildly limiting to *Thalassiosira weissflogii*) and 0.2 pM in **B** (severely limiting to *T. weissflogii*). Solid symbols represent cultures supplemented with 5 pM Cd′. No Cd was added to cultures represented by open symbols. Cultures were acclimated for one transfer in metal-defined medium at the desired Cd′ and Zn′ levels prior to experiments. The growth curves shown are for subsequent transfers. Growth rates were determined by monitoring in vivo fluorescence during exponential phase growth (regression lines are given) and normalized to maximum rates (see Table 3-1).


A. 3 pM Zn'
Tetraselmis maculata
Thalassiosira pseudonana
Thalassiosira weissflogii
Pleuorchrysis carterae
Thalassiosira oceanica
Log Fluorescence
Time (days)
5 pM Cd'
no Cd

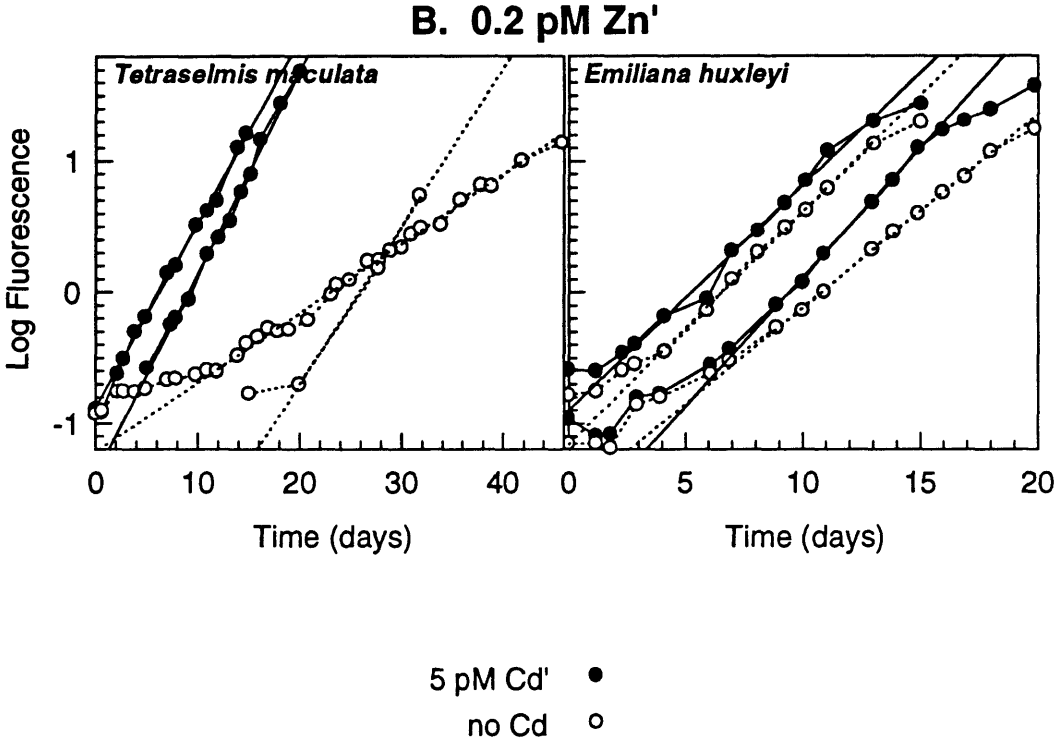

B. 0.2 pM Zn'
Tetraselmis maculata
Emiliana huxleyi
Log Fluorescence
1
0
-1
0
10
20
30
40
0
5
10
15
20
Time (days)
Time (days)
5 pM Cd'
no Cd

Table 3-1. List of species of marine phytoplankton in this study.

Division, clone designation, place of origin of the sample from which each species was isolated, size, and optimum growth rates in doublings/day are provided.

Class	Species	CCMP number	Clone	Cell vol.[1] (μm^3)	Place of Origin[2]	Maximum growth rate (doub./d)
Bacillariophyceae	*Thalassiosira pseudonana*	1015	3H(UW)	71	Long Island, NY	2.6[a]
	Thalassiosira oceanica	1005	13-1	75	Sargasso Sea	1.8[b]
	Thalassiosira weissflogii	1336	Actin	800	Long Island, NY	2.6[c]
Prymnesiophyceae	*Emiliana huxleyi*	373	BT6	50	Sargasso Sea	1.0[b]
	Pavlova lutheri	1325	Mono	75	Finland	2.2[c]
	Pleurochrysis carterae	645	CoccoII	630	Woods Hole, MA	2.7[c]
Chlorophyceae	*Dunaliella tertiolecta*	1320	Dun	350	coastal (origin unknown)	1.9[c]
	Tetraselmis maculata	897	TTM	425	Departure Bay, WA	1.6[c]
Dinophyceae	*Heterocapsa pygmaea*	1322		600	Galveston, TX	0.8[c]

[1] Cell volumes are from Ahner et al. in press except for *T. pseudonana*, which was measured by J.R. Reinfelder (pers. comm.).
[2] Places of origin were obtained from the Provasoli-Guillard Center for the Cultures of Marine Phytoplankton except for that of *T. maculata*, which was given by G. Wickfors (pers. comm.).
[a] (Sunda & Huntsman 1992)
[b] Maximum growth rates from this study.
[c] (Ahner et al. in press)

Chapter 4 Export of Cadmium and Phytochelatin by the Marine Diatom *Thalassiosira weissflogii.*[*]

J. G. Lee, B. A. Ahner, and F. M. M. Morel

Abstract

Cadmium is one of the most toxic of trace metals and induces high concentrations of the metal-binding polypeptide phytochelatin, $(\gamma\text{-Glu-Cys})_n\text{Gly}$ where $n \geq 2$, in the marine diatom *Thalassiosira weissflogii.* Here we show that at high inorganic cadmium concentrations, there is an efflux of cadmium from *T. weissflogii* so large that over half the cadmium taken up by the cell is returned to the medium. At high inorganic cadmium, there is also an efflux of phytochelatin from the cell. The efflux of both cadmium and phytochelatin stops when the external inorganic cadmium concentration is reduced. The efflux of phytochelatin and cadmium occurs at a molar ratio of approximately 4 γ-Glu-Cys subunits per cadmium, a stoichiometry similar to that measured in vivo for the cadmium-phytochelatin complex. We hypothesize that *T. weissflogii* exports the phytochelatin-cadmium complex as a detoxification mechanism. The cadmium-phytochelatin complex does not appear to be very stable in seawater once outside the cell since the cadmium exported is available to *T. weissflogii* and induces phytochelatin synthesis. Cadmium-phytochelatin export may be an important adaptive strategy which allows phytoplankton to survive in metal polluted waters.

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[*] This chapter is a modified version of Lee, J. G., B. A. Ahner, F. M. M. Morel. Submitted. Export of Cadmium and Phytochelatin by the Marine Diatom *Thalassiosira weissflogii. Environ. Sci. Technol.*

Introduction

Cadmium is one of the most toxic trace metals and is found in high concentrations in wastes from zinc smelting, electroplating and sewage treatment. In animals, the main biochemical response to Cd stress is the production of metallothionein, a primary gene product with a high cysteine content, whose role is to complex the free metal. Instead of metallothionein, algae and higher plants synthesize low molecular-weight, cysteine-rich polypeptides known as phytochelatins. Phytochelatins are a family of oligomers with the structure $(\gamma\text{-Glu-Cys})_n\text{Gly}$, where n varies from 2 to 11 (1, 2). They are synthesized from glutathione (which has the structure of phytochelatin with n=1), by the enzyme γ-glutamylcysteine dipeptidyl transpeptidase ("phytochelatin synthase") in response to high concentrations of a wide variety of trace metals and some metalloid oxyanions. Because phytochelatin synthase is activated by high free cellular metal concentrations, phytochelatin production is self-regulating (3, 4).

Cadmium is the most effective trace metal for inducing production of phytochelatin in marine phytoplankton. Phytochelatin appears to play a role in metal storage as well as detoxification since low levels are produced by many species of phytoplankton even at inorganic Cd (Cd′) concentrations far below those that impede growth (5). At Cd′ concentrations above 0.5 nM, phytochelatin reaches millimolar concentrations in *T. weissflogii* and increases rapidly with Cd′. Cadmium quotas, on the other hand, are much more constant resulting in cellular phytochelatin to Cd ratios of over 100 at nanomolar Cd′ concentrations (6).

Phytochelatin is induced very rapidly in *T. weissflogii* when exposed to Cd. Removing conditions of Cd stress results also in rapid restoration of low, basal phytochelatin levels in the cell. The goal of this study is to measure the kinetics of changes in cellular phytochelatin and Cd in order to understand the physiological response of marine phytoplankton to Cd stress and the possible effects of this response on the chemistry of natural waters.

Methods

Growth conditions for resuspension and DTPA experiments

Cultures were grown in synthetic ocean water prepared according to the recipe for Aquil (7) with the following modifications: inorganic Co was omitted from the recipe and Cd was added at a level of 4.6 nM inorganic Cd. The total EDTA concentration was 10 μM. Inorganic trace metal concentrations, M′, were calculated from total concentrations using the computer program MINEQL (8). Details of medium preparation are described in Lee et al. (9).

Cultures, inoculated from stocks maintained in autoclaved, metal-sufficient Aquil medium lacking Cd, were acclimated for one transfer in the metal-defined medium described above prior to all experiments. For experiments, parallel cultures, one with added ^{109}Cd and one without any radiolabel, were inoculated from the acclimated culture and grown at 4.6 nM Cd′. Cultures were incubated at 21°C under a constant photon flux density of 90-100 $\mu E \cdot m^{-2} \cdot s^{-1}$ (for resuspension experiments) and 70 $\mu E \cdot m^{-2} \cdot s^{-1}$ (for the DTPA experiment) in acid-cleaned polycarbonate bottles. Cell concentration was measured by electronic particle counting using a Coulter counter for unlabeled cultures and a hemocytometer for radiolabeled cultures.

Two types of experiments were performed to change the Cd′ concentration in the medium. In the first set of experiments, new media were prepared according to the above recipe but with 100 μM EDTA and no ^{109}Cd. One batch of the new medium contained 4.6 nM Cd′ and the other had no added Cd. Cells from each of the parallel cultures (radiolabeled and unlabeled) described above were harvested by gentle (<5" Hg) vacuum while in mid-exponential growth, washed with a small volume of filtered seawater, and resuspended in the new media. Subsamples for total particulate ^{109}Cd, total particulate phytochelatin, and cell concentration were taken periodically.

In the second type of experiment, Cd′ was reduced by adding a solution of 1 mM diethylenetriaminepentaacetic acid (DTPA) in sterile synthetic seawater to yield a final DTPA concentration of 85 ± 3 μM in the medium. Complexation by DTPA

reduced the Cd′ concentration from 4.6 nM to 7 pM. Subsamples for total particulate ^{109}Cd, cellular Cd fractionation, total particulate phytochelatin, and cell concentration were taken periodically.

Total particulate ^{109}Cd concentrations

An aliquot of a culture of ^{109}Cd radiolabeled cells was poured out under a laminar flow hood. From this subsample, two samples were taken to measure total particulate ^{109}Cd. Particulate material was collected on 3 μM polycarbonate filters, washed with 1 mM DTPA to remove surface ^{109}Cd (*9*), and counted using a Beckman LS1801 liquid scintillation counter. The cell concentration of the original subsample was determined by counting cells stained with Lugol's solution in a hemocytometer.

Total particulate phytochelatin concentrations

Duplicate samples were also taken from unlabeled cultures to measure total particulate phytochelatin. Each sample was filtered under gentle vacuum (<5" Hg) on to a GF/F filter and frozen in liquid N_2 for later analysis. The cell concentration of the original subsample was determined by electronic particle counting using a Coulter counter. Phytochelatin concentrations were determined by homogenizing the sample, derivatizing with the fluorescent sulfur-specific tag monobromobimane, and separating derivatized phytochelatin oligomers for detection by HPLC (*5*).

Cellular Cd fractionation

The rate at which the fractionation of Cd between the cell cytoplasm and cell membrane changed in response to lower Cd′ was assessed. From the same subsample as the total particulate ^{109}Cd samples, two samples were taken for cellular Cd fractionation, washed with DTPA as described above, resuspended in 1 mL filtered seawater, and frozen at -70°C for later analysis. Samples were thawed, the filter removed, and ground for two minutes at 23,500 rpm in a Brinkman Polytron® PT 3000 homogenizer at 4°C. The membrane and cytoplasmic fractions of the cell were

separated by centrifuging at 16,000 x g for 20 minutes. The supernatant was decanted and the pellet was washed with an additional 1 mL filtered seawater. Pellet, supernatant and wash were counted in a Beckman LS1801 scintillation counter.

Export of phytochelatin

The possibility that Cd was being exported as a phytochelatin complex was explored by directly measuring phytochelatin export. Cells were acclimated in two transfers of Aquil medium containing 4.6 nM Cd′ without inorganic Co (and without ^{109}Cd) and resuspended in fresh medium containing either no Cd or 4.6 nM Cd′ (as was done in the resuspension experiments measuring particulate Cd and phytochelatin described above). At 0, 6 and 21 hours following resuspension, cells were filtered from the medium under gentle vacuum (<5" Hg) using a GF/F filter. The filtrate was acidified to pH ~1 with methanesulfonic acid and applied to a Waters Sep-Pak C18 cartridge. The cartridge was rinsed with 10 mM methanesulfonic acid. Phytochelatin was eluted with 50 mM sodium acetate, acidified and derivatized as described above.

Bioassay for speciation of released Cd

In order to assess whether the Cd released was in an organically complexed form, the amount of phytochelatin produced by T. weissflogii was used as a bioassay for Cd′ in the medium. Conditioned synthetic ocean water was prepared by resuspending cells grown in Aquil with 4.6 nM Cd′ in synthetic ocean water for 6 hours, filtering out the cells, and freezing the filtrate for later use. After thawing, the conditioned medium was split into two aliquots and 2.5 nM Cd was added to one aliquot. Three additions were made to fresh synthetic ocean water: 2.5 nM Cd, 10 nM EC* of synthetic phytochelatin (n=2, MIT Biopolymers Laboratory), and a mixture of 10 nM EC phytochelatin (n=2) and 2.5 nM Cd. When both Cd and phytochelatin were added, phytochelatin and Cd were first dissolved in methanesulfonic acid in a small

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* EC = γ-Glu-Cys subunits = 2 x (phytochelatin, n=2) + 3 x (phytochelatin, n=3) + 4 x (phytochelatin, n=4)

volume. Seawater was added to raised the pH of the mixture, then the mixture was added to synthetic ocean water. Controls to which neither Cd nor phytochelatin were added were prepared for both fresh and conditioned medium.

Cultures were grown in Aquil medium containing 10 μM EDTA without inorganic Co or Cd. Cells were harvested during exponential growth and resuspended into the six different types of media described above. At 6.25 hours and 7.5 hours following resuspension, samples of total particulate phytochelatin were taken and cell concentration was measured.

Results

Cultures of the marine diatom *T. weissflogii* were grown at 4.6 nM Cd′, a relatively high but "sub-toxic" Cd concentration at which cells take up large amounts of Cd and produce high levels of phytochelatin, but are still able to grow at maximum rates. Three types of experiments on the kinetics of change of cellular Cd and phytochelatin concentrations were performed on these cultures: cells uniformly labeled with ^{109}Cd were resuspended in new medium containing the same Cd′ concentration but without ^{109}Cd; ^{109}Cd labeled cells were resuspended in medium without Cd; and the chelating agent DTPA was added to reduce Cd′ in the medium of ^{109}Cd labeled cultures. Each of these experiments was also performed in parallel with unlabeled cells for phytochelatin measurements. In a separate experiment, the efflux of phytochelatin from cells into the medium was measured directly. Finally, the speciation of the Cd released from the cells was studied using the induction of phytochelatin as a bioassay.

Resuspension at 4.6 nM Cd′

After resuspension in medium at the same Cd′ concentration, cultures continued to grow exponentially without any lag (Figure 4-1 A). Growth rates after resuspension changed only slightly from the initial rate (decreased by 16% for the unlabeled cultures and increased by 4% for the radiolabeled cultures, Table 4-1) and were the same (within 10%) for radiolabeled and unlabeled parallel cultures.

As shown in Figure 4-1 B, during the first 1.3 hours following resuspension, there was a small increase in the total particulate ^{109}Cd corresponding to ~1 amol Cd/cell, presumably due to uptake of radiolabeled Cd associated with the cell surface. Previous measurements of Cd uptake rates (*9*) predict that as much as 8 amol/cell could have been taken up during that time. Similar uptake of Fe associated with the cell surface has been observed (10). We therefore took our initial value for the amount of total particulate ^{109}Cd inside the cells to be that at 1.3 hours. Over the next 11 hours of the experiment, the rate of ^{109}Cd efflux decreased with the total particulate ^{109}Cd and

became practically null while a significant fraction of the initial ^{109}Cd (13 nCi/L) still remained in the cells. Total particulate phytochelatin increased steadily over the course of the experiment, indicating that phytochelatin was being produced constantly as cells grew (Figure 4-1 C).

The concentration of ^{109}Cd per cell also decreased rapidly due to efflux of Cd from the cell and also, though more slowly, due to dilution by growth (Figure 4-1 B). As expected since the external Cd′ concentration was unchanged by resuspension, the concentration of phytochelatin per cell remained constant (Figure 4-1 C), providing evidence that all other cellular parameters (e.g. the cellular Cd concentration, the Cd uptake rate, and the fractionation of Cd between membrane and cytoplasmic proteins) also remained at steady-state as expected.

Resuspension in medium without Cd and addition of DTPA

After resuspending cells in medium without any Cd, growth continued exponentially (Figure 4-2 A) and at a similar rate (6% slower for unlabeled cells and 6% faster for radiolabeled cells, Table 4-1), although the initial cell concentration measurement following resuspension of ^{109}Cd labeled cells was high, likely due to inadequate mixing. Growth also continued exponentially after DTPA addition (Figure 4-3 A) and at rates only slightly less than the initial rate of the unlabeled culture (rates decreased by 10% for unlabeled cells and by 21% for radiolabeled cells, Table 4-1). The initial growth rate for radiolabeled DTPA cultures was most likely overestimated due to the large error associated with the hemocytometer cell concentration measurements. Growth rates of radiolabeled and unlabeled cultures were within 6% of each other in the resuspension experiment and within 7% of each other in the DTPA experiment. In the DTPA experiment, growth rates were slower than in the resuspension experiment due to low light levels (70 vs. 100 $\mu E \cdot m^{-2} \cdot s^{-1}$, see Methods).

When Cd′ in the medium was reduced by resuspension in medium without Cd, total particulate Cd initially increased by a small amount (~0.2 amol Cd/cell, Figure 4-2 B), similar to what happened in the previous resuspension experiment (Figure 4-1 B).

When Cd′ was reduced by adding DTPA, total particulate Cd began to decrease immediately (Figure 4-3 B). As in the washing technique used to measure intracellular ^{109}Cd (*9*), DTPA rapidly removed any Cd bound to the surface of the cells, preventing initial uptake of surface-associated Cd. In both the DTPA and resuspension without Cd experiments, the rate of total particulate Cd efflux was rapid at first but stopped altogether after a period of time (Figure 4-2 B and Figure 4-3 B). After DTPA addition, efflux of total particulate Cd continued for much longer (~8 hours) and was a much larger fraction of the initial value (3.0 nM) than when cells were suspended in medium without added Cd (efflux stopped after ~3 hours, initial total particulate Cd was 1.2 nM). The faster response to lower external Cd′ in the resuspension experiment may have been due to more rapid growth. The total particulate phytochelatin concentration either remained constant during efflux of total particulate Cd (Figure 4-2 C) or increased slowly (Figure 4-3 C). After total particulate Cd efflux stopped, total particulate phytochelatin concentrations declined in both experiments.

The apparent cessation of net efflux of total particulate Cd when cells were resuspended in medium without Cd could result from a balancing of efflux from the cell by uptake of radiolabeled Cd back into the cells. Phytochelatin concentrations per cell continued to decline, however, and the complexation kinetics of the released Cd by EDTA should be quite rapid at an EDTA concentration of 100 μM (99% complexed in 3 minutes, 11) making the released Cd unavailable to the cell.

In both the resuspension and DTPA experiments, the Cd concentration per cell decreased rapidly initially and, of course, decreased more slowly when the efflux of total particulate Cd stopped (Figure 4-2 B and Figure 4-3 B). The initial Cd and phytochelatin concentrations per cell were higher for the DTPA experiment because of unusually large cell size (Figure 4-3 B and C). While total particulate Cd was decreasing, the concentration of phytochelatin per cell either decreased slowly (Figure 4-2 C) or remained constant (Figure 4-3 C). After the efflux of total particulate Cd stopped, the concentration of phytochelatin per cell decreased rapidly. The difference in the rates at which the concentrations of Cd and phytochelatin per cell were reduced

resulted in a constant (Figure 4-2 D) or increasing (Figure 4-3 D) cellular phytochelatin to Cd ratio while there was an efflux of total particulate Cd and a rapid decrease in phytochelatin:Cd after the Cd efflux stopped.

Changes in cellular Cd fractionation

After DTPA was added, the total particulate Cd pool decreased from an initial value of 3.0 nM over the first 8 hours and then remained constant at 2.1 nM (Figure 4-4 A). The distribution of Cd between the cytoplasm and membrane components of the cell changed over the same time period, with an average of 20% of cellular Cd being associated with membrane material initially and 35% membrane-associated at the end of the experiment (Figure 4-4 B). Steady-state values for cell fractionation are 21% membrane-associated at 4.6 nM Cd′ and 40% at 7 pM Cd′, indicated by the arrows on the left and right axes respectively of Figure 4-4 B (*9*). As a result of this change in fractionation and Cd efflux, the amount of Cd in the total cytoplasmic pool decreased from 2.4 to 1.4 nM while the membrane-bound pool increased slightly from 0.6 nM to 0.7 nM (Figure 4-4 A).

Efflux of phytochelatin

The efflux of phytochelatin into the medium was also measured directly following resuspension of cells conditioned to 4.6 nM Cd′ in EDTA-buffered medium containing the same Cd′ concentration or no added Cd (Figure 4-5). Initially, some phytochelatin was detected in the medium indicating a small amount of cell breakage, but the initial phytochelatin concentration was only 5.5 nM EC* of n = 2, 3 and 4 phytochelatin (0.7% of the initial total particulate phytochelatin concentration) and 7.5 nM EC (0.9% of the initial total particulate phytochelatin concentration) for cells resuspended at 4.6 nM Cd′ and no Cd, respectively. In cultures resuspended at 4.6 nM Cd′, the concentration of phytochelatin in the medium increased linearly over the

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* EC = γ-Glu-Cys subunits of phytochelatin = 2 x (phytochelatin, n=2) + 3 x (phytochelatin, n=3) + 4 x (phytochelatin, n=4)

course of the experiment, reaching 30 nM EC after 21.5 hours. In cultures resuspended in medium without Cd, the amount of dissolved phytochelatin in the medium initially increased the same amount as at 4.6 nM Cd′ (9 vs. 8 nM EC of phytochelatin during the first 6 hours) but increased by only 4 nM EC of phytochelatin during the subsequent 16 hours of the experiment. In comparison, the efflux of total particulate Cd was 0.1 nM Cd from cells resuspended in medium without Cd over 12 hours (Figure 4-2 B) and 0.9 nM Cd from cells in the 15 hours following DTPA addition (Figure 4-3 B).

Bioassay for speciation of released Cd

Like other physiological responses to Cd exposure such as growth rate, the amount of phytochelatin produced by cells depends on the Cd′ concentration rather than the total Cd concentration. The phytochelatin response can therefore be used to assay how much uncomplexed Cd is present in the culture medium. To assay the speciation of Cd released into the medium by the cells, synthetic ocean water without EDTA was conditioned by adding cells grown at 4.6 nM Cd′ to it for 6 hours and then filtering out the cells (see Methods). New cells grown in medium without Cd (but with Aquil trace metals and EDTA) were then transferred into the conditioned medium and, for comparison, fresh synthetic ocean water (without EDTA), to which Cd, phytochelatin and a mixture of the two had been added (see Methods). The initial concentration of phytochelatin per cell was 29 amol EC*/cell. This level remained essentially unchanged in cells resuspended in fresh synthetic ocean water whether or not 10 nM EC of synthetic phytochelatin was also added (Table 4-2). Adding 2.5 nM Cd (all of which is present in an inorganic form) to fresh synthetic ocean water produced elevated phytochelatin levels of ~120 amol EC/cell after 6 hours, again regardless of the presence or absence of 10 nM EC of phytochelatin in the medium. Conditioned synthetic ocean water, which contained 16 nM EC initially, produced

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* EC = γ-Glu-Cys subunits of phytochelatin = 2 x (phytochelatin, n=2) + 3 x (phytochelatin, n=3) + 4 x (phytochelatin, n=4)

cellular phytochelatin concentrations comparable to those produced by adding 2.5 nM Cd to fresh synthetic ocean water (100 amol EC/cell). This indicates a Cd′ concentration, uncomplexed by phytochelatin, in the conditioned medium of a bit less than 2.5 nM Cd′. According to our previous data on the rate of efflux of Cd by cells conditioned in the same Cd′ medium and resuspended in medium without Cd, we expected a release of between 0.1 and 1 nM Cd (depending on how quickly export was shut off). Adding an additional 2.5 nM Cd as inorganic Cd to conditioned synthetic ocean water produced the highest cellular phytochelatin concentrations, 360 amol EC*/cell after 6 hours, over three times higher than those produced by the same Cd concentration in fresh synthetic ocean water. Duplicates collected at 7.5 h following resuspension were essentially the same (Table 4-2).

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* EC = γ-Glu-Cys subunits of phytochelatin = 2 x (phytochelatin, n=2) + 3 x (phytochelatin, n=3) + 4 x (phytochelatin, n=4)

Discussion

We measured a significant efflux of both Cd and phytochelatin from *T. weissflogii* cells grown at 4.6 nM Cd′. Several lines of evidence indicate that this efflux is actually export under physiological control rather than the result of cell breakage or leakage: 1) cell growth continues after resuspension without any lag phase and at the same rate; 2) the Cd efflux is observed when Cd′ is reduced by adding DTPA, which, unlike filtration, has no potential to cause physical damage to the cells; and 3) the Cd and phytochelatin efflux stops or slows dramatically when the external Cd′ concentration is reduced (either by adding DTPA or resuspending cells in medium without Cd) as compared to when cells remain at a high Cd′ concentration. The simplest way to explain our data is to hypothesize that Cd and phytochelatin are exported as a Cd-phytochelatin complex via a mechanism which is under physiological control. Below we examine the coherence of this hypothesis with the results of the our various experiments.

Cd export rate

To analyze our data quantitatively, we assumed that there are two pools of Cd in the cell, a labile pool (presumably the Cd-phytochelatin complex) which is available for export and a non-labile pool (Cd bound to proteins) which is not exported. This is the simplest model consistent with our result that the amount of ^{109}Cd exported approaches an asymptotic value that is only a fraction of the total particulate ^{109}Cd concentration. As cells grow at a rate μ, Cd from the labile pool is exported with a first order rate constant κ and incorporated into the non-labile pool with a first order rate constant λ. The labile pool is replenished by uptake of Cd from the medium. The equations describing changes in the labile pool, non-labile pool, and cell concentration over time are given in Table 4-3. When cells are exposed to a steady-state external Cd′ concentration, the Cd concentration per cell remain constant ($\frac{dQ_N(t)}{dt} = 0$, see Table 4-3). As a result, f, the fraction of intracellular Cd in the non-labile pool, λ, the rate

constant for partitioning into the non-labile pool, and μ, the specific growth rate, are related to each other by:

$$f = \frac{\lambda}{\mu + \lambda}$$

If all of the rates remain constant when uptake of ^{109}Cd is stopped (by resuspension in cold medium or addition of DTPA), the amount of exported ^{109}Cd at a given time is given by:

$$H(0) - H(t) = \frac{\kappa}{(\kappa + \lambda)}(1 - f) \cdot H(0)\left[1 - e^{-(\kappa+\lambda)t}\right]$$

where H(t) is the total particulate ^{109}Cd concentration in nCi/L.

By fitting the data on Cd efflux in Figure 4-1 B and Figure 4-3 B with a non-linear least squares optimization algorithm (omitting the last measurement in the DTPA experiment, see below) we obtain the values for κ of 0.16 h^{-1} from the data in Figure 4-1 B and 0.11 h^{-1} from the data in Figure 4-3 B. The values for λ are 0.071 h^{-1} from Figure 4-1 B and 0.025 h^{-1} from Figure 4-3 B (Table 4-4). It was fortuitous that μ varied to the extent that it did in our experiments, providing two different data sets from which to calculate the rate constants. The resulting values for f , 0.51 and 0.49, agree quite well supporting the interpretation of the Cd export data provided by the model. The results of the model for exported ^{109}Cd (given above) are plotted in Figure 4-1 B, Figure 4-2 B and Figure 4-3 B as dashed lines for the period over which Cd is exported (the values for κ and λ from resuspension in medium with 4.6 nM Cd′ were also used for resuspension in medium without Cd). After the phytochelatin to Cd ratio reaches a maximum value (Figure 4-2 D and Figure 4-3 D), we assume no more Cd is exported and the "model" becomes a horizontal line.

The first order rate constant for Cd export calculated above is consistent with previous measurements of Cd uptake (*9*). The rate of uptake, ρ, must balance export and dilution to maintain a constant concentration of Cd per cell, Q:

$$\rho = \mu Q + \kappa(1-f) Q$$

From this equation and the results of the resuspension experiment in Table 4-4, for every 4 atoms of Cd taken up into the cell, 2 are exported and two are incorporated into cell material, one each in the labile and non-labile pools. When cells are growing slower as in the DTPA experiment, for every 6 atoms of Cd taken up, 4 are exported and two are incorporated into cell material, again one each in the labile and non-labile pools. We have previously determined that uptake rates of Cd by *T. weissflogii* follow Michaelis-Menten kinetics where ρ_{max} = 0.17 amol·cell^{-1}·min^{-1} and K_m = 2900 pM for cells conditioned at 0.46 nM Cd′ (*9*). Using these constants, ρ would be 6.3 amol/cell-h at 4.6 nM Cd′. From the equation for ρ given above, the rate of Cd uptake at the initial Cd′ concentration of 4.6 nM is calculated to be 5.6 amol·cell^{-1}·h^{-1} from the resuspension experiment and 6.1 amol·cell^{-1}·h^{-1} from the DTPA experiment (Table 4-4), in good agreement with the expected value for ρ from the Michaelis-Menten constants.

Changes in Cd fractionation between the membrane and cytoplasm

This model of the kinetics of Cd efflux and partitioning in the cell also allows us to calculate the expected change in fractionation between cytoplasmic and membrane proteins following addition of DTPA. From the results of the model, initially 51% of the cellular Cd was in the labile pool (hypothesized to correspond to the pool of Cd complexed intracellularly by phytochelatin) and the remaining 49% of the cellular Cd was incorporated into the non-labile pool (hypothesized to be proteins in both the cell membrane and cytoplasm). Since initially 20% of the cellular Cd was associated with membrane proteins (Figure 4-4 B), the fractionation of Cd within the non-labile pool was 41% (20% + 49%) in membrane proteins and the balance, 59%, in cytoplasmic proteins. This value for fractionation within the non-labile pool is in good agreement with measurements of cellular Cd fractionation at low (5 pM) Cd′ where 40% of the total cellular Cd is in membrane proteins (*9*) and the labile Cd pool would be expected to be negligible. The relative rates at which labile Cd is exported and incorporated into

the non-labile pool as calculated from the model also agree reasonably well with the amount of Cd lost from the cytoplasm and gained in the membrane during the course of the DTPA experiment. The ratio of Cd incorporated into the membrane to Cd exported was 0.15 and the ratio of 41% of λ (since 41% of the non-labile pool was membrane protein) to κ was 0.09 ($41\% \times 0.025\ h^{-1} + 0.11\ h^{-1}$).

The data in Figure 4-4 support the idea that phytochelatins may also provide storage for Cd for use in proteins in addition to aiding in detoxification of Cd. At limiting Zn′ concentrations, Cd can substitute for Zn in some proteins and restore growth (*9*, 12). The model which fits our data on the dynamics of Cd within the cells suggests that the source of Cd for proteins both in the membrane and cytoplasm is the labile pool of Cd, hypothesized to be the pool of Cd complexed intracellularly by phytochelatin. Indeed, phytochelatin-metal complexes have been shown to restore the activity of apoenzymes which require metals in vitro (13).

Export of Cd as a phytochelatin complex

Both Cd and phytochelatin export stop after a similar period of time when the external Cd′ concentration is reduced by resuspending cells in medium without Cd (Figure 4-2 B and Figure 4-5). This similar timing suggests that Cd and phytochelatin export may be co-regulated. One would expect co-regulation of Cd and phytochelatin export if Cd were exported as a phytochelatin complex. Transport of Cd bound as a phytochelatin complex has been observed in other organisms and appears to confer Cd tolerance. A putative ATP-binding protein for transport of Cd-phytochelatin complexes into the cell vacuole has been sequenced in fission yeast (14). In tobacco cells, both Cd and phytochelatin accumulate in the vacuole in response to Cd toxicity (15). Further the stoichiometry of Cd and phytochelatin export is similar to that measured in Cd-phytochelatin complexes from yeast (4 moles sulfur per mole Cd, 16). From ρ (Cd uptake rate during the resuspension experiment, Table 4-4), Q_{init} (initial concentration of Cd per cell during the resuspension experiment, Table 4-4), and the following equation:

$$\text{rate of Cd export} = (\rho - \mu Q_{min}) \cdot \chi(t)$$

the ratio of phytochelatin to Cd exported is calculated to be 8 moles sulfur per mole Cd at 6 hours and 4 at 21 hours. The value at 6 hours is likely too high due to a small amount of cell breakage increasing the apparent amount of phytochelatin exported.

The Cd-phytochelatin complex does not appear to be stable once exported. As a complex, Cd would presumably not be biologically available. However, the results of the bioassay experiment (Table 4-2) indicate that the exported Cd is taken up and induces a phytochelatin response in the cell that is consistent with the Cd in the medium being uncomplexed. The affinity of phytochelatin for Cd may be insufficient for the phytochelatin-Cd complex to remain intact at the concentrations in the medium. Phytochelatin may also be oxidized to a product such as phytochelatin disulfide, which would bind Cd less strongly but still be detected as phytochelatin by the measurement technique.

Effect of Cd-phytochelatin export on natural waters

In polluted coastal waters, Cd′ concentrations can be on the order of a few nM, comparable to those used in this study. We may then expect that the rates of uptake and export of Cd from phytoplankton would also be comparable to the rates measured in this study. The cellular Cd export flux we have measured here (with a characteristic rate constant of 0.1 h^{-1}) would therefore be at least as large as fluxes associated with grazing, such as release during cell breakage and incorporation into fecal material (with characteristic rate constants on the order of 1 d^{-1}, assuming a phytoplankton population at steady-state with a growth rate of ~1 d^{-1}). If the phytochelatin to Cd ratio were as high as we have measured here, in contrast to Cd export, the cellular export of phytochelatins would presumably be only a small fraction of the release resulting from sloppy feeding of zooplankton. Our data suggest that in more pristine waters at low Cd′ concentrations, there is likely no direct export of the Cd-phytochelatin complex (although cell breakage by zooplankton grazing (17) and viral lysing could be a significant source of Cd-phytochelatin).

The exported phytochelatin does not appear to affect Cd speciation in the medium. Even if Cd-phytochelatin export does not provide phytoplankton with the ability to control environmental Cd′ concentrations, export of Cd may well be an important adaptive mechanism for regulating internal Cd′ concentrations and surviving at high metal levels.

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Figure 4-1. Resuspension in EDTA-buffered medium with 4.6 nM Cd′.

Cell concentration, total particulate ^{109}Cd, and total particulate phytochelatin were monitored over time after resuspension in EDTA-buffered medium containing 4.6 nM Cd′ without ^{109}Cd. In panel **A**, cell concentration of radiolabeled (●) and unlabeled (○) cultures are shown. Cadmium data were obtained from radiolabeled cultures and phytochelatin data were from unlabeled cultures. The measured (□) and predicted amount (dashed lines) of total particulate ^{109}Cd efflux is shown in panel **B**. The ^{109}Cd concentration per cell (■) is also plotted in panel **B**. In panel **C**, the phytochelatin concentration per cell (■) and the total particulate phytochelatin concentration (□) are given. Error bars in panel **A** represent one standard deviation above and below the mean. The error bars for the total particulate ^{109}Cd efflux represent the range of duplicate measurements.

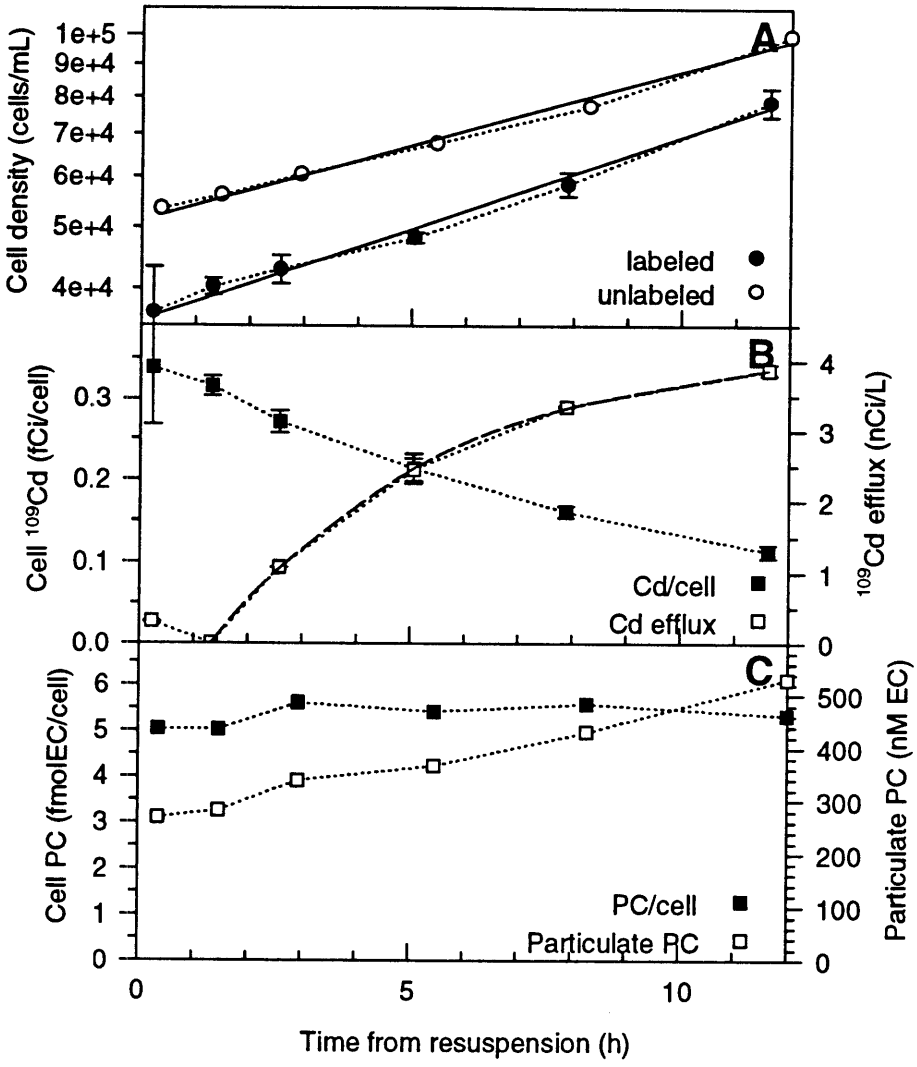
A
B
C
Cell density (cells/mL)
1e+5
9e+4
8e+4
7e+4
6e+4
5e+4
4e+4
labeled
unlabeled
Cell ^{109}Cd (fCi/cell)
0.3
0.2
0.1
0.0
^{109}Cd efflux (nCi/L)
4
3
2
1
0
Cd/cell
Cd efflux
Cell PC (fmolEC/cell)
6
5
4
3
2
1
0
Particulate PC (nM EC)
500
400
300
200
100
0
PC/cell
Particulate PC
0
5
10
Time from resuspension (h)

Figure 4-2. Resuspension in EDTA-buffered medium containing no added Cd.

Cell concentration, total particulate ^{109}Cd, and total particulate phytochelatin were monitored over time after resuspension in EDTA-buffered medium containing no added Cd. Symbols, curve fits and error bars for panels **A**, **B** and **C** are the same as described for Figure 4-1 except that in panel **B** the Cd concentration per cell and total particulate Cd efflux were calculated using the initial ^{109}Cd specific activity. From the concentration of Cd and phytochelatin per cell, the cellular ratio of phytochelatin to Cd was calculated (**D**).

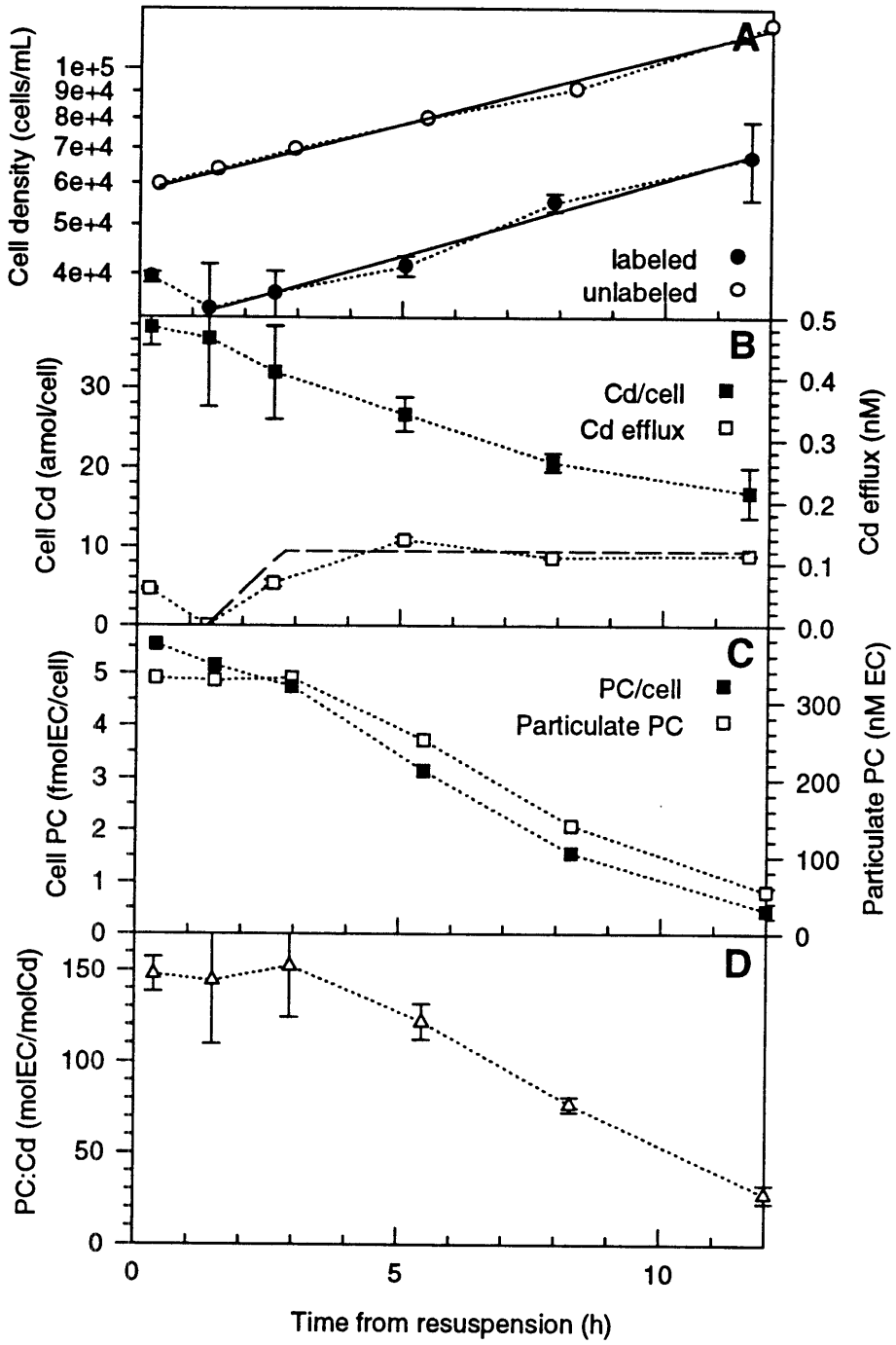
A
Cell density (cells/mL)
1e+5
9e+4
8e+4
7e+4
6e+4
5e+4
4e+4
labeled
unlabeled
B
Cell Cd (amol/cell)
30
20
10
0
Cd/cell
Cd efflux
0.5
0.4
0.3
0.2
0.1
0.0
Cd efflux (nM)
C
Cell PC (fmolEC/cell)
5
4
3
2
1
0
PC/cell
Particulate PC
300
200
100
0
Particulate PC (nM EC)
D
PC:Cd (molEC/molCd)
150
100
50
0
0
5
10
Time from resuspension (h)

Figure 4-3. Addition of DTPA to reduce Cd′.

Cell concentration, total particulate ^{109}Cd, and total particulate phytochelatin were monitored over time after addition of DTPA to reduce Cd′. Symbols, curve fits and error bars for all panels are the same as described for Figure 4-2 except that in panel **B** the Cd concentration per cell was also calculated using estimated cell concentrations (◆). Also, in panel **C** duplicate measurements of total particulate phytochelatin were made.

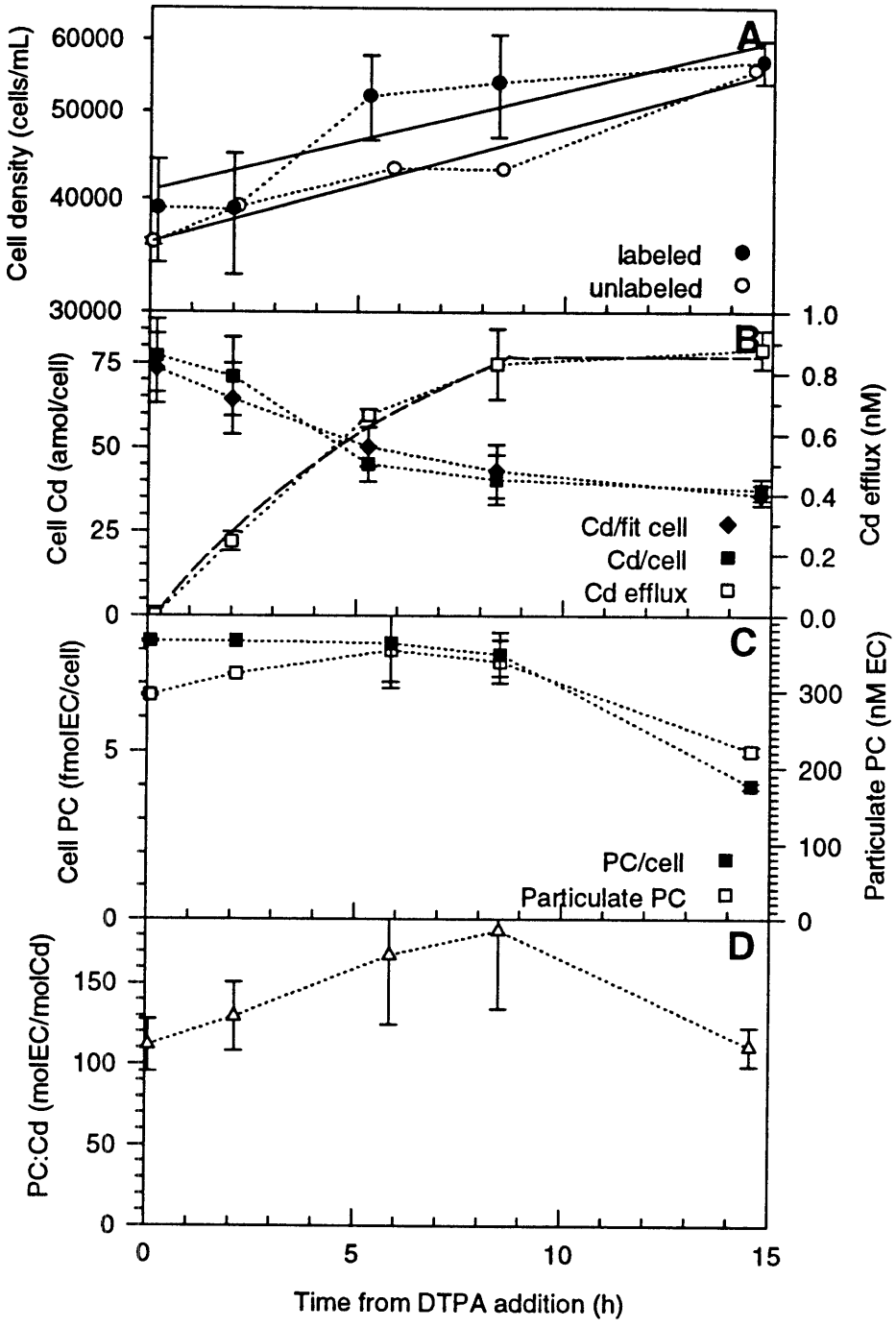
A
B
C
D
Cell density (cells/mL)
60000
50000
40000
30000
labeled
unlabeled
Cell Cd (amol/cell)
75
50
25
0
Cd/fit cell
Cd/cell
Cd efflux
Cd efflux (nM)
1.0
0.8
0.6
0.4
0.2
0.0
Cell PC (fmolEC/cell)
5
0
PC/cell
Particulate PC
Particulate PC (nM EC)
300
200
100
0
PC:Cd (molEC/molCd)
150
100
50
0
0
5
10
15
Time from DTPA addition (h)

Figure 4-4. Cadmium fractionation between membranes and cytoplasm following addition of DTPA to reduce Cd′ levels in the medium.

Total particulate Cd (**A**, ◆) and cellular fractionation of Cd (**B**) was measured following addition of DTPA to reduce Cd′ levels in the medium. Also in panel **A**, the amount of Cd associated with the cytoplasmic fraction of cells (Δ) and cell membranes (O) was calculated. Error bars represent the range of duplicate measurements. Arrows indicate steady-state cell fractionation at 4.6 nM Cd′ (left axis) and 7 pM Cd′ (right axis) from Lee et al. (9).

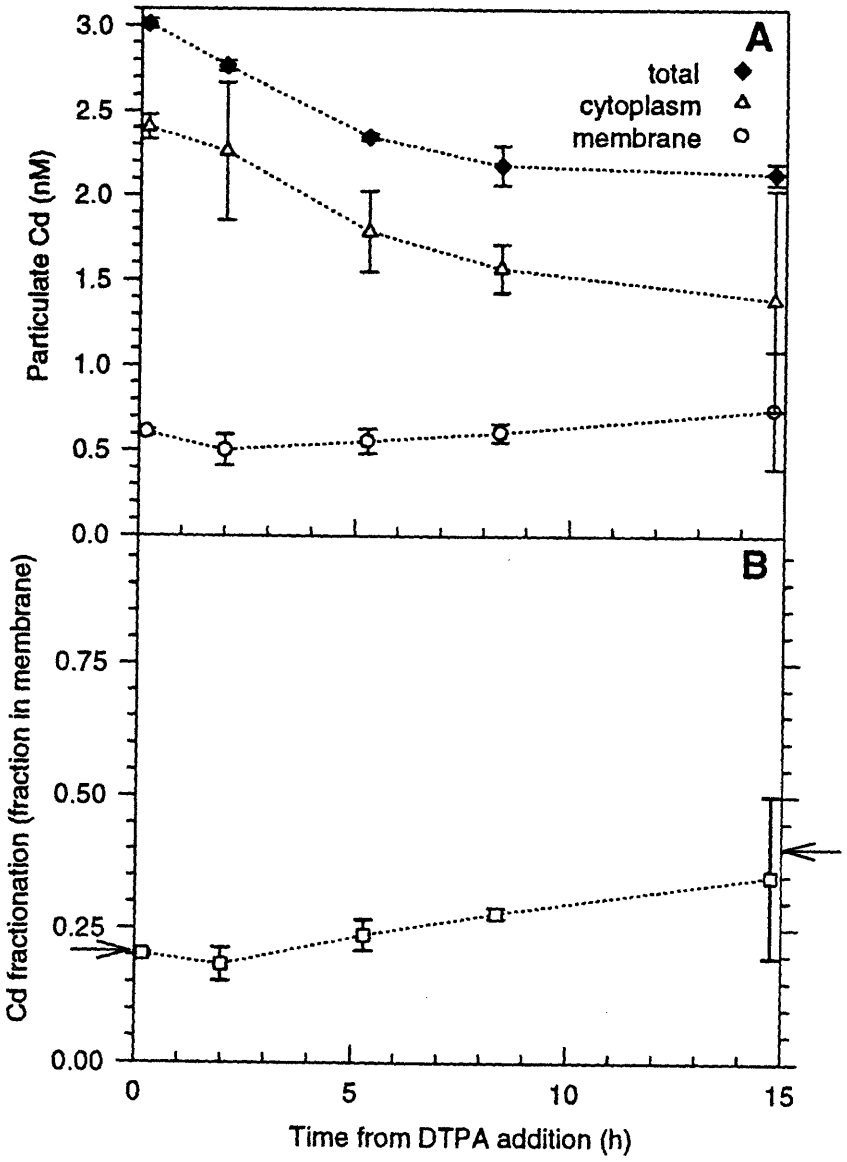
A
total
cytoplasm
membrane
Particulate Cd (nM)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
B
Cd fractionation (fraction in membrane)
0.75
0.50
0.25
0.00
0
5
10
15
Time from DTPA addition (h)

Figure 4-5. Efflux of phytochelatin from cells following resuspension in EDTA-buffered medium with and without Cd.

Dissolved phytochelatin in the culture medium was measured over time following resuspension in EDTA-buffered medium containing 4.6 nM Cd′ (●) and no added Cd (○).

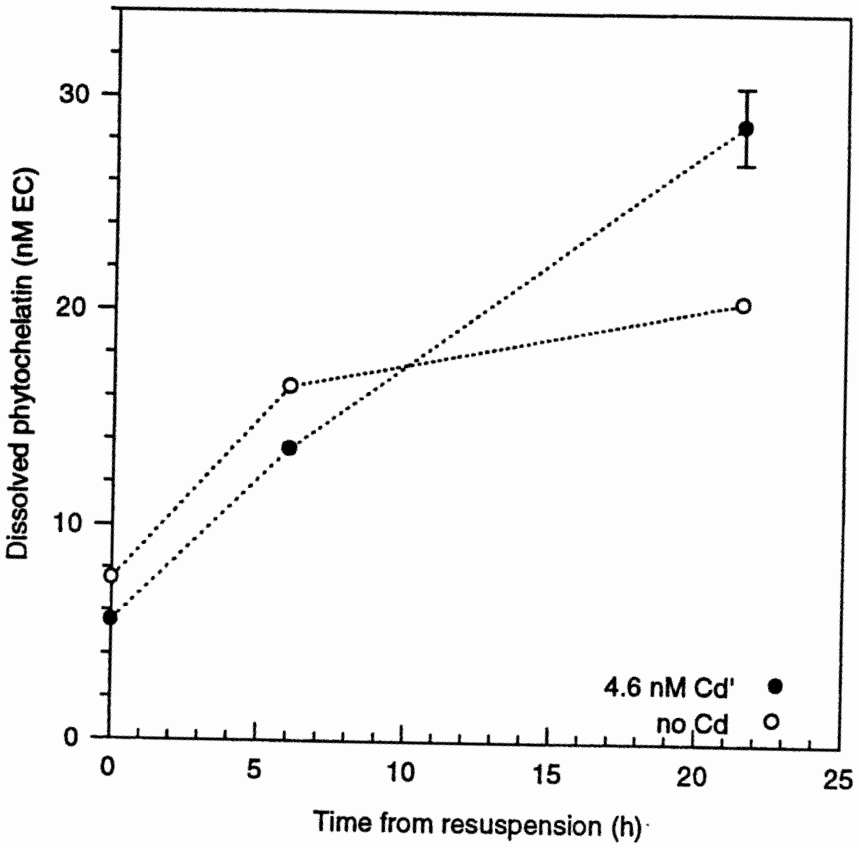
Dissolved phytochelatin (nM EC)
30
20
10
0
0
5
10
15
20
25
Time from resuspension (h)
4.6 nM Cd'
no Cd

Table 4-1. Growth rates of cultures before and after change in Cd′

Experiment	Time	^{109}Cd	Cd′ (nM)	Growth Rate (d^{-1})	Growth Rate (doubling·d^{-1})
Resuspension	before	no	4.6	1.5	2.2
	before	yes	4.6	not meas.	not meas.
	after	no	4.6	1.3	1.9
	after	yes	4.6	1.6	2.3
	after	no	~0	1.4	2.1
	after	yes	~0	1.6	2.3
DTPA addition	before	no	4.6	0.78	1.1
	before	yes	4.6	1.1	1.5
	after	no	~0	0.70	1.0
	after	yes	~0	0.61	0.88

Table 4-2. Results of bioassay for speciation of released Cd

Resuspension Medium	Phytochelatin added (nM)	Cd added (nM)	Phytochelatin induced (amol EC·cell^{-1}) 6.25 h	7.5 h
Fresh	none	2.5	130	130
	none	none	32	28
	5	2.5	110	120
	5	none	37	31
Conditioned	none	2.5	360	450
	none	none	101	89

Table 4-3. Model of efflux of total particulate ^{109}Cd and change in Cd per cell

Variable	Definition	Units	Equation
$\frac{d H_L(t)}{dt}$	change in labile fraction of total particulate ^{109}Cd	$\frac{nCi}{L \cdot h}$	$-(\kappa+\lambda)H_L(t)$
$\frac{d H_N(t)}{dt}$	change in non-labile fraction of total particulate ^{109}Cd	$\frac{nCi}{L \cdot h}$	$\lambda H_L(t)$
$\frac{d \chi(t)}{dt}$	change in cell concentration	$\frac{cells}{L \cdot h}$	$\mu \chi(t)$
$\frac{d Q_L(t)}{dt}$	change in labile Cd per cell	$\frac{amol}{cell \cdot h}$	$\rho-(\kappa+\lambda+\mu)Q_L(t)$
$\frac{d Q_N(t)}{dt}$	change in non-labile Cd per cell	$\frac{amol}{cell \cdot h}$	$\lambda Q_L(t)-\mu Q_N(t)$

Table 4-4. Rate constants for Cd efflux and cellular partitioning

Experiment	κ (h^{-1})	λ (h^{-1})	f	ρ (amol Cd·cell^{-1}h^{-1})	Q_{int} (amol Cd·cell^{-1})
Resuspension	0.16	0.071	0.51	5.6	38
DTPA addition	0.11	0.025	0.49	6.1	73

Chapter 5 Cadmium in carbonic anhydrase: changes with PCO_2.

Introduction

Recent work has shown that Cd can replace Zn in the marine diatom *T. weissflogii* (Lee et al. in press) and in other species of marine algae (Lee and Morel in press). Supplementing Zn-limited cultures of these algae with Cd allows rapid growth, although it does not always restore growth to the full rates of Zn-sufficient cultures. We have also shown that Cd restores activity of the enzyme carbonic anhydrase (CA) in Zn-limited cultures and that Cd coelutes with some of the isoforms of CA produced in *T. weissflogii* (Lee et al. in press). The enzyme CA contains almost all of the cellular Zn in *T. weissflogii* (Morel et al. 1994). It is therefore likely that the principal mechanism by which Cd enhances growth in *T. weissflogii* is replacement of Zn by Cd in carbonic anhydrase and that thus Cd is involved in carbon acquisition.

Here we test the hypothesis that Cd has a role in carbon acquisition by examining the effect of varying PCO_2 on CA activity and Cd distribution among the cellular proteins of *T. weissflogii*.

Methods

Growth conditions

Cultures of *T. weissflogii* (Center for the Culture of Marine Phytoplankton, Bigelow Laboratory) were grown in synthetic ocean water prepared according to the recipe for Aquil (Price et al. 1988/89) with the following modifications: inorganic Co was omitted from the recipe since Co, like Cd, can substitute for Zn (Price & Morel 1990); inorganic Zn was reduced to 3 pM instead of the normal Aquil level of 16 pM; and Cd was added at a level of 46 pM inorganic Cd. Carrier-free ^{109}Cd (New England Nuclear) was also added to the medium at a concentration of 1.1 μCi/L. Inorganic trace metal concentrations, M′, were calculated from total concentrations using the computer program MINEQL (Westall et al. 1976). Details of media preparation are described in Lee et al. (in press).

Cultures were inoculated from stocks maintained in autoclaved, metal-sufficient Aquil medium and acclimated at a given PCO_2 in one transfer of Zn-limiting medium with Cd prior to all experiments. Cultures were grown in acid-washed, polycarbonate bottles, incubated at 26°C under a constant photon flux density of 130 $\mu E \cdot m^{-2} \cdot s^{-1}$. The desired PCO_2 level was achieved by bubbling at a rate of 1-2 bubbles/s with 0.2 μM filtered air containing 100, 300 or 1000 ppm CO_2 (Middlesex Gases). In the experiment on the steady-state effects of PCO_2, a pair of cultures were grown in medium labeled with ^{109}Cd and a parallel pair of cultures was grown without any radiolabel for phytochelatin measurements. Growth was monitored by *in vivo* fluorescence (^{109}Cd labeled cultures) or electronic particle counting using a Coulter counter (unlabeled cultures).

Short-term effects of lowering PCO_2

A single, ^{109}Cd labeled culture was grown at 1000 ppm PCO_2 for the experiment on the short-term effects of lowering PCO_2. In mid-exponential growth, the PCO_2 was reduced to 100 ppm. At the same time the chelating agent

diethylenetriaminepentaacetic acid (DTPA) dissolved in sterile, synthetic ocean water run through a chelex column (Price et al. 1988/9) was added for a final DTPA concentration of 87 μM. Cell concentration and cellular Cd quotas were measured at 0.1, 3.2 and 6.8 hours following reduction of PCO_2.

Cadmium quotas

Cells from ^{109}Cd labeled cultures were harvested by filtering them under gentle vacuum (< 5" Hg) onto a 3 μm polycarbonate membrane filter. Metal associated with the cell surface was removed by incubating for 10 minutes in a 1 mM solution of diethylenetriaminepentaacetic acid (DTPA) in filtered seawater and washing with small volumes of filtered seawater as described in Lee et al. (in press). The activity of the cells on the filter was determined using a Beckman LS1801 liquid scintillation counter. The cell concentration was determined by counting cells stained with Lugol's solution in a hemocytometer. Cell quotas were calculated using the specific activity of the medium and the cell concentration at the time of measurement. Duplicate measurements were performed on each culture.

Cadmium fractionation

Cultures were grown and harvested as described above for Cd quotas. Cells washed with DTPA as for Cd quotas and were resuspended in 1000 μL filtered seawater and cells were frozen at -70°C. To determine partitioning between membrane-bound and soluble Cd in the cell, samples were thawed, the filter was removed, and cells were ground for two minutes at 23.5K rpm in a Brinkman Polytron PT 3000 homogenizer at 4°C. The membrane and cytoplasmic fractions of the cell were separated by centrifuging at 16,000 x g for 20 minutes. The supernatant was decanted and the pellet was washed with an additional 1 mL filtered seawater. The pellet was dissolved in a small volume of 95% ethanol and brought to a 1 mL volume with filtered seawater. The dissolved pellet, supernatant and wash were counted in a Beckman LS1801 scintillation counter. Triplicate samples were taken from each

culture (although only the measurements from one culture have been completed and are presented in Figure 5-1).

Cellular phytochelatin concentration

Cells from unlabeled cultures were filtered under gentle vacuum (<5" Hg) onto a GF/F filter and frozen in liquid N_2 for later analysis. The cell concentration was determined by electronic particle counting using a Coulter counter. Phytochelatin concentrations were determined by homogenizing the sample, derivatizing with the fluorescent sulfur-specific tag monobromobimane, and separating derivatized phytochelatin oligomers for detection by HPLC (Ahner et al. in press). Duplicate samples were taken from each culture (although only the measurements from one culture have been completed and are presented in Figure 5-1).

Carbonic anhydrase assay

From one of the radiolabeled cultures, cells were harvested as described for Cd quotas. They were resuspended in 10 mL filtered seawater, pelleted by centrifugation for 10 minutes at 1400 g in a Beckman TJ-6 centrifuge with a swinging bucket rotor, frozen at –70°C and stored at –20°C for analysis. Cells were later thawed and a small volume (300 µL) of breaking buffer containing anti-proteases (100 mM NaCl/20 mM NaH_2PO_4/10 µM leupeptin/1 mM PMSF, pH 7.0) was added. Cell suspensions were ground for four minutes at 23.5K rpm in a Polytron PT 3000 homogenizer at 4°C. Unbroken cells and cellular debris were removed by centrifuging at 16,000 g in a Eppendorf 5415C centrifuge with a fixed angle rotor for 20 minutes at 4°C. The supernatant was decanted, frozen in liquid N_2, and stored at –20°C.

Carbonic anhydrase activity was detected using a post-electrophoresis enzyme assay on samples containing equal amounts of ^{109}Cd activity (2.1 nCi). Non-denaturing polyacrylamide gel electrophoresis was carried out according to the Laemmli method (Sambrook et al. 1989) on a 10% polyacrylamide gel. The gel was soaked in a solution of 0.1% bromcresol purple in fresh electrophoresis buffer, blotted dry and placed in a

pure CO_2 atmosphere until red or yellow bands of CA activity appeared against the purple background. The gel was then frozen on dry ice and photographed with a Wratten 74 green filter under long wave UV light using Polaroid Instant black and white film (Morel et al. 1994).

A ^{109}Cd elution profile was determined from adjacent lanes containing the same samples. Autoradiography was performed on the dried gel using a two week exposure and intensifying screens.

Results and Discussion

Growth rates (Figure 5-1 A), the concentration of Cd per cell (Figure 5-1 B) and the fractionation of Cd between the membrane and cytoplasm in the cell (Figure 5-1 C) vary little at high, atmospheric, and low CO_2 levels. Growth rates increased slightly with PCO_2 levels from 1.3 at 100 ppm to 1.7 doubling/d at 1000 ppm (only the increase in growth rate between 100 ppm and 300 ppm is significant at the 90% confidence level, Table 5-1). These rates were higher than those of Zn-limited cultures without Cd (0.8 doubling/d at 3 pM Zn′ and atmospheric CO_2, Lee et al. in press), but cultures were not able to grow quite as rapidly as they would with sufficient Zn′ (1.8 doubling/d at atmospheric CO_2, Lee et al. in press), however.

Cellular Cd concentrations increased slightly with PCO_2 from 16 amol Cd/cell at low CO_2, to 17 amol Cd/cell at atmospheric CO_2, to 18 amol Cd/cell at high CO_2 (Figure 5-1 B). This increase with increasing PCO_2 is significant at the 95% confidence level between 300 and 100 ppm and at the 90% confidence level between 1000 and 300 ppm. These values agree fairly well with previous measurements of 23 amol Cd/cell at the same Zn′ and Cd′ levels and atmospheric CO_2 (Lee et al. in press). We did not expect cellular Cd concentrations to increase with CO_2 since the Cd requirement for CA would be highest at low PCO_2. In fact, normalized to fluorescence rather than cell concentration, the cellular Cd concentration decreased slightly with increasing PCO_2 (Figure 5-1 B) because the fluorescence per cell increased with increasing PCO_2, although only the difference between 1000 and 100 ppm is statistically significant at the 90% confidence level (Table 5-1).

The fraction of cellular Cd associated with the membrane decreased slightly with increasing PCO_2 (Figure 5-1 C; but this decrease is not significant, Table 5-1). The percent of Cd associated with the membrane fraction of the cell, 15-21%, was significantly lower than previous measurements of ~40% (Lee et al. in press), attributable to poor recovery of the pellet after washing with seawater (see Methods).

There was a dramatic difference in the cellular phytochelatin concentration with PCO_2 (Figure 5-1 D; significant at the 95% confidence level, Table 5-1). At low PCO_2

cells contained 1.1 fmol EC*/cell, almost three times as much as was present at high PCO_2. As a result of the large change in cellular phytochelatin concentration, the ratio of phytochelatin to Cd in the cell varied from 22 at high PCO_2 to 73 at low PCO_2.

Although total cellular Cd concentrations and Cd fractionation was little changed by PCO_2, the amount of Cd associated with CA did vary. *T. weissflogii* produces up to 7 different apparent CA isoforms and/or subunits, only some of which coelute with Cd (Lee et al. in press). In this study, only the two most active isoforms were detected (Figure 5-2). Low PCO_2 increased the activity of both isoforms including the main band of CA activity where Co and Zn coelute† (Morel et al. 1994, Yee et al. in prep.). There was a corresponding increase in the amount of Cd which coeluted with the upper isoform of CA (Figure 5-3). Little Cd coeluted with the lower CA isoform, but a significant amount of Cd coeluted with the dye front. Phytochelatin also coelutes with the dye front (data not shown).

The short-term effects of simultaneously lowering PCO_2 and external Cd′ were in accord with the steady-state observations described above. The growth rate was not much affected (Figure 5-4 A). Before the PCO_2 was reduced, cultures were growing at 1.7 doubling/d and afterwards the culture grew at 1.3 doubling/d (the low cell concentration at 3.2 hours was likely due to poor mixing of the culture before sampling).

The initial cellular Cd concentration, 15 amol Cd/cell (Figure 5-4 B) was somewhat lower than in the previous experiment (Figure 5-1 B). Cellular Cd concentration decreased over time between 3 and 7 hours due to dilution by growth. The amount of total particulate Cd exported was 0.03 nM in 7 hours (Figure 5-4 C), much less than the 0.6 nM that was exported during the same time period from cells

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* EC = γ-Glu-Cys subunits of phytochelatin = 2 x (phytochelatin, n=2) + 3 x (phytochelatin, n=3) + 4 x (phytochelatin, n=4)

† CO_2 diffused in from the edge of the gel making the activity of the main CA band in lane H appear equal to that in lanes M and L, whereas prior to freezing (see Methods), the activity in the main CA band appeared lower in lane H than in lanes M and L, which had approximately equal activity.

grown at 4.6 nM Cd′ (Lee et al. in review). The change in total particulate Cd is not significant at the 90% confidence level (Table 5-1).

The variation of Cd coeluting with the upper isoform of carbonic anhydrase confirms that Cd is being used in carbonic anhydrase. Restoration of CA activity of Zn-limited cultures by adding Cd *in vivo* also provides indirect evidence that Cd is used in CA (Morel et al. 1994, Lee et al. in press). This upper isoform of CA may be designed specifically for Cd rather than Zn since almost all of the cellular Zn coelutes with the lower CA isoform (Morel et al. 1994, Yee et al. in prep.).

Previously, we have shown that the intracellular pool of Cd-phytochelatin complex can supply Cd to proteins when the external Cd′ concentration is reduced (Lee et al. in review). Should phytochelatin be playing the role of supplying Cd for protein synthesis, one might expect to see a depletion of Cd bound to phytochelatin at low PCO_2 levels. The amount of Cd associated with the dye front (and by implication, with phytochelatin) does not appear to vary with PCO_2 however. It is likely that the Cd concentration in this study was too high to observe changes in the Cd-phytochelatin pool, Cd being abundant enough that a large surplus could be stored bound to phytochelatin, with only a small fraction of the stored pool being used in carbonic anhydrase at low PCO_2.

A reasonable model for Cd metabolism can be developed to account for these data on the effect of PCO_2 and our previous results. At low steady-state values of PCO_2, Cd is taken up more rapidly by the cell because more Cd is needed for CA. This uptake may be through a specific Cd transporter or through the Zn transporter, which should also be enhanced under these conditions. Higher phytochelatin concentrations per cell are then produced at low PCO_2 because of the increase in cellular Cd. Phytochelatin-bound Cd is used as a source to synthesize a specific isoform of CA. If this specific CA were subsequently exported to the periplasmic space for uptake of bicarbonate, it would explain why cellular Cd concentrations are slightly lower at lower PCO_2. If the model is correct, Cd may be exported actively from the cell at either very

low Cd′ (if needed for a periplasmic CA) or at very high concentrations as a detoxifying system.

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Figure 5-1. Growth, cellular Cd, cellular phytochelatin, and Cd fractionation at varying PCO2.

Duplicate cultures were grown at atmospheric (300 ppm), low (100 ppm) and high (1000 ppm) PCO_2 in Zn-limited medium (3 pM Zn′) containing 46 pM Cd′. Growth rates were monitored (**A**) and cells were harvested in late exponential growth for measurements of cellular Cd concentration (**B**), fractionation of Cd between the membrane and cytoplasm (**C**) and cellular phytochelatin concentration in fmol EC*/cell (**D**). The ratio of cellular phytochelatin to cadmium concentrations was also calculated in panel **D**. Error bars represent the range of average values for duplicate cultures (**B**, **D**) or the standard deviation of replicate measurements (**A**, **C**).

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* EC = γ-Glu-Cys subunits of phytochelatin = 2 x (phytochelatin, n=2) + 3 x (phytochelatin, n=3) + 4 x (phytochelatin, n=4)

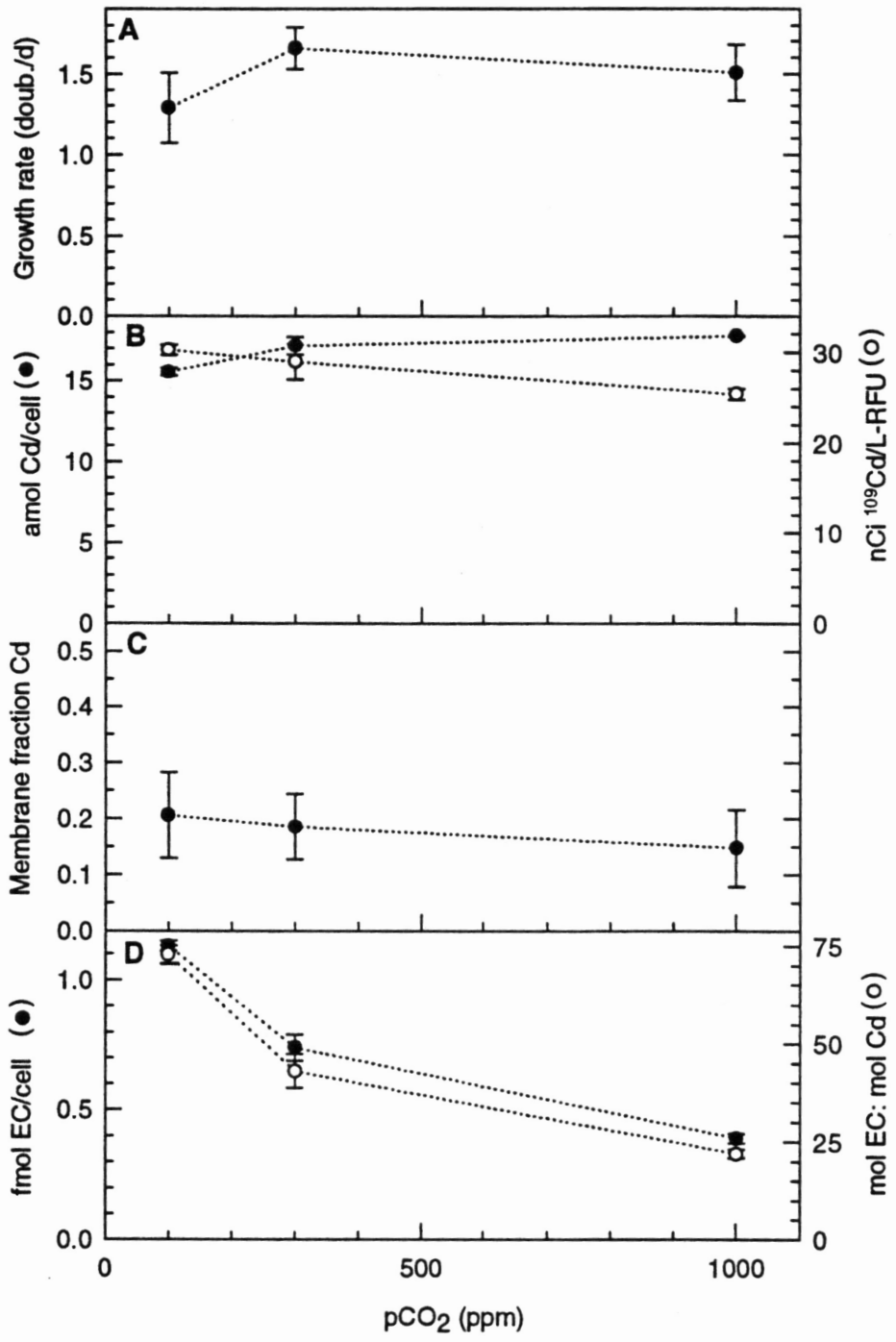
A
B
C
D
Growth rate (doub./d)
amol Cd/cell (●)
nCi ^{109}Cd/L-RFU (o)
Membrane fraction Cd
fmol EC/cell (●)
mol EC: mol Cd (o)
pCO$_2$ (ppm)
0
500
1000

Figure 5-2. CA activity at varying P_{CO_2}.

Cultures were grown at high (**H**, 1000 ppm), atmospheric (**M**, 300 ppm) and low (**L**, 100 ppm) P_{CO_2} in Zn-limited medium (3 pM Zn′) containing 46 pM radiolabeled Cd′. The carbonic anhydrase activity of cell extract samples containing equal ^{109}Cd activity was assayed post-electrophoresis using a pH sensitive fluorescent dye. Regions of CA activity appear white against the dark background.

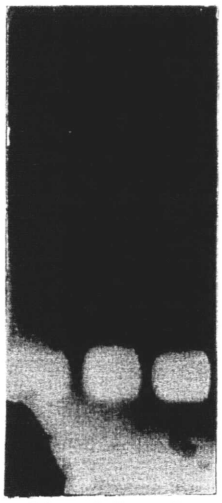

Figure 5-3. Cd content of CA at varying P_{CO_2}.

Cultures were grown at high (**H**, 1000 ppm), atmospheric (**M**, 300 ppm) and low (**L**, 100 ppm) P_{CO_2} in Zn-limited medium (3 pM Zn′) containing 46 pM radiolabeled Cd′. Autoradiography was performed post-electrophoresis on the dried gel. The location of CA activity (from Figure 5-2) and the dye front is indicated by bars on the right side of the gel frame and arrow, respectively.

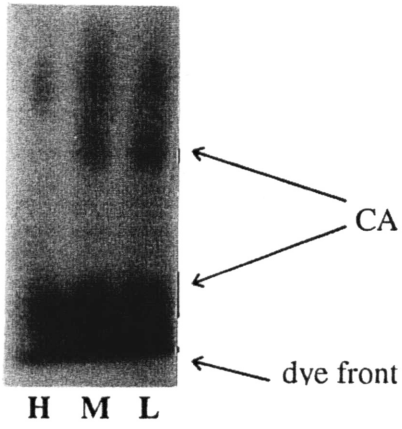

Figure 5-4. Short term CO_2 effects on growth, cellular Cd, and total particulate Cd.

Cultures were grown in Zn-limited medium (3 pM Zn′) at high PCO_2 (1000 ppm) and switched to low PCO_2 (100 ppm). At the same time Cd′ was reduced from 46 pM to 7 pM by the addition of DTPA. Cell concentration (**A**), cellular Cd concentrations (**B**), and total particulate Cd (**C**) were measured after 0.1, 3.2 and 6.8 hours at low PCO_2.

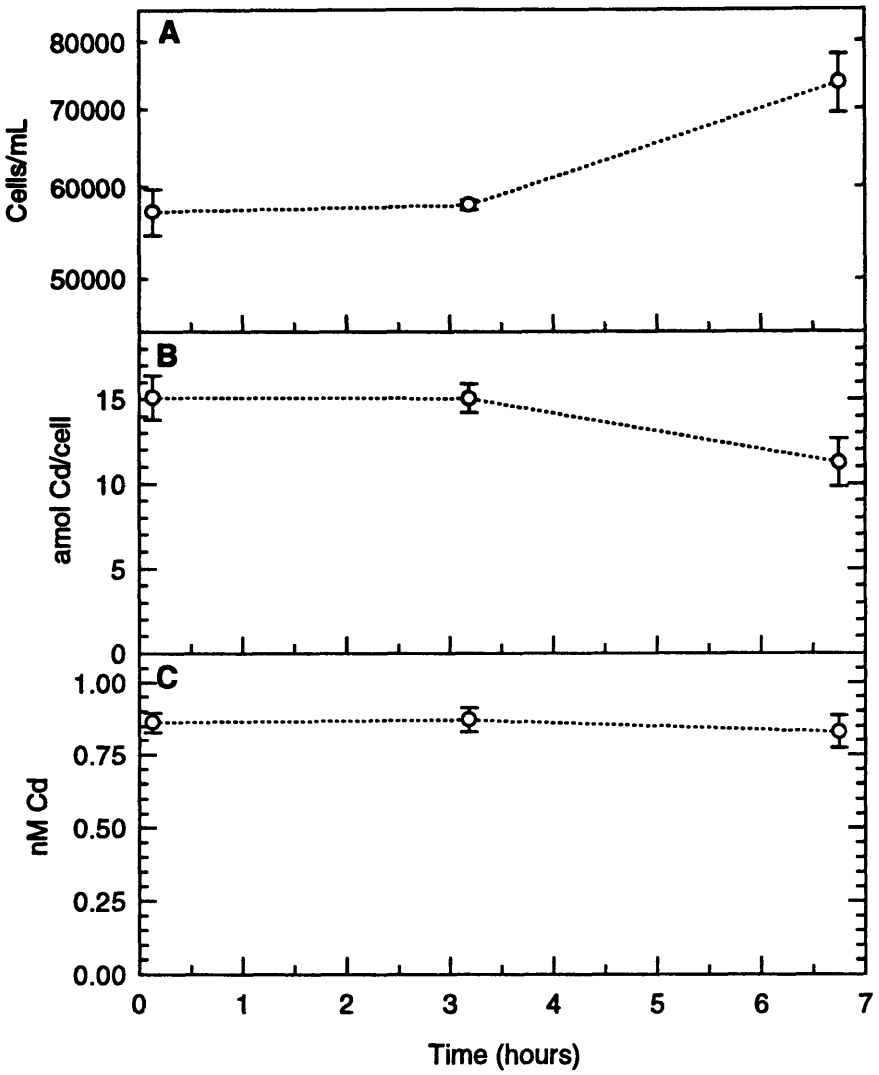
A
80000
70000
60000
50000
Cells/mL
B
15
10
5
0
amol Cd/cell
C
1.00
0.75
0.50
0.25
0.00
nM Cd
0
1
2
3
4
5
6
7
Time (hours)

Table 5-1. Statistical significance of changes in parameters measured at steady-state (with varying P_{CO_2}) and after lowering Cd′ (and reducing the P_{CO_2}).

Parameter	Samples compared	Figure	Significant at confidence level	
			90%	95%
Growth rate	1000, 300 ppm	Figure 5-1	no	no
	1000, 100 ppm	Figure 5-1	no	no
	300, 100 ppm	Figure 5-1	yes	yes
Cd quota	1000, 300 ppm	Figure 5-1	yes	no
	1000, 100 ppm	Figure 5-1	yes	yes
	300, 100 ppm	Figure 5-1	yes	yes
$nCi \cdot RFU^{-1}$	1000, 300 ppm	Figure 5-1	no	no
	1000, 100 ppm	Figure 5-1	yes	yes
	300, 100 ppm	Figure 5-1	no	no
Membrane-associated Cd	1000, 300 ppm	Figure 5-1	no	no
	1000, 100 ppm	Figure 5-1	no	no
	300, 100 ppm	Figure 5-1	no	no
Cellular phytochelatin*	1000, 300 ppm	Figure 5-1	yes	yes
	1000, 100 ppm	Figure 5-1	yes	yes
	300, 100 ppm	Figure 5-1	yes	yes
Total particulate Cd	0, 3 hours	Figure 5-4	no	no
	0, 7 hours	Figure 5-4	no	no
	3, 7 hours	Figure 5-4	no	no

* Standard deviation of phytochelatin measurements was estimated from range of duplicates.

Chapter 6 DTPA wash method for measuring intracellular Cd

Rationale used to develop method

Trace metals can bind non-specifically to the surface of phytoplankton cells. A technique to remove surface-associated Cd with a solution of the chelating agent diethylenetriaminepentaacetic acid (DTPA) was developed. Similar techniques have been developed to measure intracellular nickel (Price and Morel 1991) and iron (Hudson and Morel 1989). Preliminary experiments to determine intracellular Cd by washing with seawater solutions containing Na_2EDTA and α-hydroxyquinoline produced unsatisfactory results: the measured Cd concentration per cell actually increased with increasing wash time. Most likely the Cd-chelator complex was able to diffuse through the cell membrane. DTPA was selected as a better chelating agent for this technique since the Cd-DTPA complex would be more highly charged and the kinetics of complexation of Cd would be faster than EDTA in the presence of Ca (Hering and Morel 1988).

The length of time for the DTPA wash was chosen by comparing the measured cellular Cd concentration with successively longer wash times (Figure 6-1). Wash times of longer than 10 minutes did not appear to remove more ^{109}Cd from the surface of the cell. The amount of surface-associated Cd removed by the DTPA wash technique was estimated to be 2 to 4 amol Cd/cell from a comparison of the measured cellular Cd concentration with a 2 min and 6-12 min DTPA wash. The initial cellular Cd concentration appears to be non-zero because the Cd was not equilibrated with EDTA prior to the experiments (unlike all other experiments in previous chapters, see Figure 2-3 for example).

The DTPA concentration used was the highest which was readily soluble in seawater, dissolving completely when left unstirred overnight. Filtered seawater was used as a solvent to minimize osmotic shock to the cells.

Details of Method

Samples of cells radiolabeled with $^{109}Cd^{*}$ are harvested by filtering them under gentle vacuum (<5" Hg) onto a 3 μm polycarbonate membrane filter (Poretics). The filter apparatus used is a 25 mm DELTAWARE® unit (VWR Scientific) with a glass filter support. Before adding the sample to the filter unit, a small amount of filtered seawater is added to cover the filter and wet the tower-support interface (minimizing contamination of the filter unit by the radiolabeled sample). The tubing leading from the bottom of the filter unit to the vacuum is clamped so that flow can be started and stopped (a trap is inserted before the pump to collect the filtrate). The flow is stopped with liquid just above the filter during all washes of the sample to minimize cell breakage due to drying.

Following filtration of the culture medium containing the cells to just above the filter, ~5 mL of 1 mM DTPA in filtered seawater is added to the sample. The DTPA wash remains in contact with the cells for 10 minutes with the flow stopped.

Following the DTPA wash, the sample is then washed 3 times with ~5 mL of filtered seawater. On the final wash, the filter is allowed to go to dryness. The radioactivity of the cells on the filter is determined by putting the whole filter in Optifluor scintillation fluid and counting the sample with a Beckman LS1801 liquid scintillation counter. No quench correction is included in converting cpm to dpm since quenching of ^{109}Cd is negligible.

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* The specific activity was adjusted by adding cold Cd as well as carrier-free ^{109}Cd (Amersham) to the culture medium so that the measured radioactivity per sample would be approximately 1000 cpm.

References

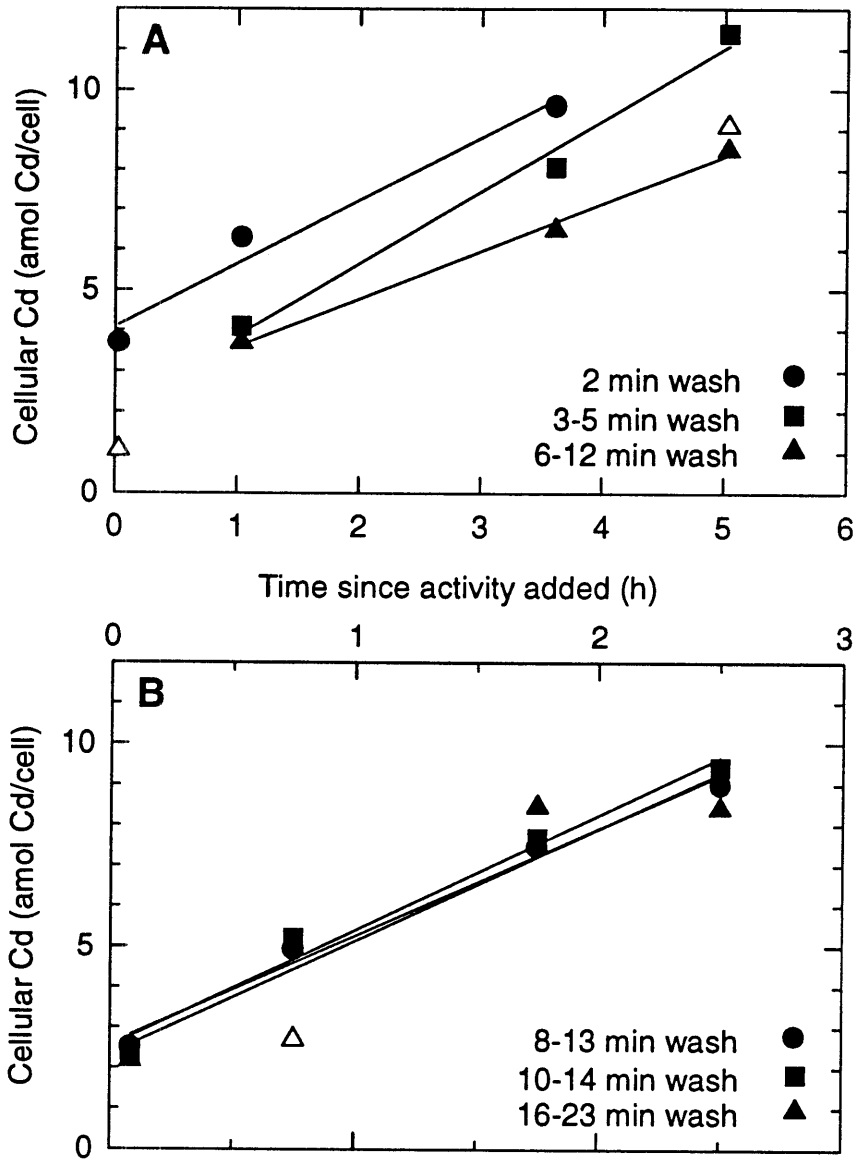
Hering, J. G. and F. M. M. Morel. 1988. Kinetics of trace metal complexation: Role of alkaline-earth metals. Environ. Sci. Technol. 22: 1469-1478.

Hudson, R. J. and F. M. M. Morel. 1989. Distinguishing between extra- and intracellular iron in marine phytoplankton. Limnol. Oceanogr. 34: 1113-1120.

Price, N. M. and F. M. M. Morel. 1991. Colimitation of phytoplankton growth by nickel and nitrogen. Limnol. Oceanogr. 36: 1071-1077.

Figure 6-1. The effect of increasing DTPA wash times on measured cellular Cd concentration.

Cells were grown in Aquil-type medium without any ^{109}Cd. A mixture of radiolabeled Cd, cold Cd and EDTA (equimolar to the Cd concentration) was added at t=0. The ^{109}Cd activity was measured over time as described. The cellular Cd concentration was calculated using the ^{109}Cd specific activity and cell concentration. The duration of the DTPA wash ranged from 2 to 12 minutes in panel A and 8 to 23 minutes in panel B (different cultures were used in panels A and B but the Cd′ levels and specific activity were the same). Open symbols are inaccurate measurements where cells adhered to the filter unit while the filter was being removed.


A
Cellular Cd (amol Cd/cell)
10
5
0
0
1
2
3
4
5
6
2 min wash
3-5 min wash
6-12 min wash
Time since activity added (h)
B
Cellular Cd (amol Cd/cell)
0
1
2
3
10
5
0
8-13 min wash
10-14 min wash
16-23 min wash